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COOPERATIVE AGREEMENT NUMBER: DAMD17-93-V-3008

TITLE: Autoradiographic Distribution and Applied Pharmacological Characteristics of Dextromethorphan and Related Antitissue/Anticonvuleant Drugs and Novel Analogs

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REPORT DATE: October 1995

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Final 27 Sep 93 - 26 Sep 95

4. TITLE AND SUBTITLE
Autoradiographic Distribution and Applied Pharmacological Characteristics of Dextromethorphan and Related Antitissue/ Anticonvuleant Drugs and Novel Analogs

5. FUNDING NUMBERS
DAMD17-93-V-3008

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Accesion For	
NTIS CRA&I	☒
DTIC TAB	☒
Unannounced	☐
Justification	
By	
Distribution /	
Availability Codes	
Dist	Avail and / or Special
A-1	

14. SUBJECT TERMS
Dextromethorphan, Drugs, CD

15. NUMBER OF PAGES
72

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Contents

1.0 Introduction

1.1 Historical background

Relief of pain has always been one of the great objectives in medicine, and in this regard morphine has been for a long time an invaluable tool in the hand of the practitioner.

However, the severe side effects with which this substance is endowed have often limited its use. This explains why such an effort was made during the first half of the century in the synthesis of new derivatives that could have been capable of exerting an analgesic effect comparable to that of morphine without side effects such as addiction liability, respiratory depression, or toxicity. Modifications of morphine`s polycyclic structure have led to one of the first series of structure-activity relationship for this class of substances and to a high number of derivatives with different degrees of selectivity for different opioids receptors.

Martin et al. (1976) first attempted to distinguish between the action of opioids at different opioid receptors.

Making use of the chronic spinal dog, Martin and coworkers attributed three different syndromes produced by congeners of morphine to three distinguishable receptors (μ, κ, σ). Morphine produced myosis, bradycardia, hypothermia, general depression of the nociceptive responses and indifference to environmental stimuli acting at μ receptors; ketocyclazocine

constricted pupils, depressed flexor reflex and produced sedation through κ receptors; SKF 10.047 (n-allyl-normetazocine) caused mydriasis, tachypnea, tachycardia and mania acting at σ receptors. All these compounds induced tolerance and were reported to be antagonized by naloxone.
Further studies identified the presence of a fourth class of receptors, named δ, and demonstrated that different endogenous peptides have different affinities for these subclasses of opioid receptors (Lord et al. 1977).

Dextromethorphan (DM) was first prepared by Schnider and Grussner in 1951, between a series of compounds derived from morphine. Many studies were then conducted in both humans and rodents to define the pharmacological profile of this derivative (Benson et al. 1952, Randall et al 1950).

1.2 Dextromethorphan and opioids

In a comparatory study of the pharmacology of levorphan, racemorphan and DM and their related methylethers, Benson et al. (1953) extended the results already found by Isbell et al (1953) in human: levorotatory isomers of dextrorphan and dextromethorphan are endowed with analgesic activity and addiction liability comparable to that exerted by morphine, and are able to induce an abstinence syndrome; but these actions, as well as the respiratory depressant and miotic activity, were not shared by the dextrorotatory isomer.

Moreover, the methylated compound was found to be less potent but more active orally than the demethylated form.
Both isomers were found to be effective in raising the cough threshold in human and animals, but with the dextrorotatory isomer this action was devoid of any lethargy or ataxia (Benson et al. 1953).
The effectiveness of DM in elevating the cough threshold and the absence of any opioid-like side effects is the basis for the utilization of this drug as a cough suppressant.
But even though DM acts in a similar manner to that of codeine to elevate the cough threshold (Hahn et al. , 1966), it is doubtful whether DM interacts with the same receptor as the opioids in suppressing cough since DM has a different conformation from the opioids which are stereospecific in their action.
Indeed, if DM and codeine were exerting their antitussive activity through the same receptor, this receptor should lack the stereospecificity associated with the other pharmacological actions of opiates and would therefore represent a distinct subclass of opiate binding sites.
Furthermore, Cavanagh et al. (1976) have reported that naloxone up to 40 mg/kg is unable to reverse the antitussive activity of DM, while it typically reverses that of codeine.
Although codeine and DM appear to share the same morphine-

like structure and have antitussive activity, they do not seem to mediate their actions through the same receptor.

1.3 Dextromethorphan and sigma receptors

Over the years, the original definition by Martin of the σ receptor has been revised, due to the heterogeneity of the drugs belonging to the group typified by N-allylnormetazocine (SKF 10.047). It is now known that SKF 10.047 binds in rat brain to at least two distinct binding sites (Sircar et al. 1986, Largent et al. 1986): a high affinity site (Kd 42nM) resembling the σ receptor labelled with ^{3}H-(+)-3-(-3-hydroxyphenyl)-N-(1-propyl)piperidine (^{3}H-(+)PPP), potently inhibited by (+)PPP, haloperidol and pentazocine and stereoselective for the (+) isomer of SKF 10.047; and the lower affinity binding site (Kd 615 nM) similar to the PCP receptor where PCP, TCP and m-NH_2-PCP are potent inhibithors (Largent et al. 1986). No stereoselectivity was noted for the low affinity binding site.

The rank order of potency of drugs displacing SKF 10.047 from low affinity binding sites (Sircar et al.,1986) corresponds to the one reported for PCP sites in brain (Vincent at al.,1979), while the selectivity pattern for the high affinity binding site (haloperidol>SKF 10.047>pentazocine>PCP) do not correspond to any known behaviour.

Animal studies have not clearly defined which site is responsible for the behaviour induced by the administration of σ ligands, but the different degrees of stereoselectivity shown by the effect of SKF 10,047 in squirrel monkey and pigeons (Katz et al. 1985), and rats (Shannon et al. 1982), and the antagonism of SKF 10.047 as a discriminative stimulus by haloperidol (Cone et al. 1984, Steinfels et al 1985) strongly suggest a

behavioural regulation by SKF 10.047 at σ sites.

Craviso et al. (1980) first described the presence of a high affinity, saturable and reversible binding of ^{3}HDM to guinea pig brain homogenates.
This binding was not inhibited by opioids agonists and antagonists as e.g. morphine and naloxone, and even the non-addicting isomers (+)-methadone or (-)-propoxyphene, which were still endowed with antitussive activity but devoid of analgesic action, showed low affinity for this site.
Subsequent studies by the same author detected high (Kd 13-20 nM) and low (Kd>200nM) affinity sites for DM in guinea-pig brain homogenates (Craviso et al. 1982). Competition studies (Craviso et al. 1982b) indicated no correlation of DM binding with any of the known or putative neurotransmitters in the central nervous system. IC50 values in the nanomolar range were exhibited by some phenothiazine neuroleptics and typical and atypical antidepressants (e.g. haloperidol, imiprazine, perphenazine, amytriptiline, opipramol). A specific increase in DM binding was exerted by the anticonvulsants phenytoin and ropizine.
The introduction of computer-assisted modeling to homologous and heterologous competition studies between DM and (+)-PPP permitted Klein and Musacchio (1992) to characterize in more

detail the identity of the high and low affinity site for DM in rat brain. They confirmed the existence of a high (Kd 47 nM) and a low affinity binding site for DM, but they also highlighted the heterogeneity of the high affinity site. According to the authors this would be actually composed of a (+)PPP-sensitive subpopulation identified as a haloperidol-sensitive sigma site and of a (+)PPP non-sensitive subpopulation that represent a specific DM site.

The common sigma/DM site is ten times less abundant in rat brain compared to guinea-pig brain and represents roughly 30% of the high affinity binding of DM to this tissue. Binding to this site is enhanced by ropizine and phenytoin.

1.4 Dextromethorphan and serotonin

When Canoll et al. (1990) attempted to examine the effect of ropizine on DM binding to guinea pig brain by the use of autoradiography, the result obtained was quite unexpected: they demonstrated a biphasic effect of ropizine on DM binding in different areas of the brain.

Ropizine enhanced DM binding in the subcommissural organ, choroid plexus, cerebellum, CA3 and dentate gyrus of the hippocampus, but had an inhibitory effect on areas showing the highest levels of binding such as substantia nigra, dorsal raphe,

dorsal tegmental nucleus and geniculate body. A good correlation was found between the enhancement by ropizine and the antagonism by (+)-pentazocine. This is consistent with the hypothesis that the site enhanced by ropizine is common to DM and (+)PPP.

The identity of the second site is still unknown, but the colocalization of many of the high affinity DM-binding areas with areas containing serotoninergic cell bodies or terminals, together with the antagonism of DM binding by some antidepressant drugs which block the uptake of serotonin with high affinity (Craviso and Musacchio 1982b), would suggest a relationship with the serotoninergic system.

Evidence for this hypothesis has already appeared in the literature: firstly, DM is able to prevent the acute depletion of brain serotonin by p-chloroamphetamine (PCA) in rats (Henderson and Fuller 1992).

Following the administration of PCA in rats, short and long term effects can be distinguished. The acute effect is a non-toxic depletion of serotonin from neurons that can be prevented selectively by serotonin uptake carrier blockers. The long term effect is a neurotoxic damage to serotoninergic neurons (lasting up to 4 months after administration) that can be prevented by the means of serotonin uptake inhibitors but also by neuroprotectants such as Ca channel blockers and NMDA receptor antagonists (Finnegan et

al. 1991).

Secondly, DM has been found to increase serotonin release from the nucleus of the solitary tract (NTS) (Kamei et al. 1992),

The nucleus of the solitary tract is the site of the first central synapse for primary afferent fibers that originate from airway receptors, and the NTS plays an important role in the regulation of cough (Korpas and Tamori, 1979).

and finally, DM inhibits serotonin uptake into human blood platelets (Ahtee 1975).
In her work, Ahtee showed that DM not only exerts its effect on human platelets (IC50=120 nM), but it also decreases the levels of 5-hydroxyindoleacetic acid (5-HIAA) in rat brain.

The fall in 5-HIAA levels is an effect quite common following the administration of serotonin uptake inhibitors. One possible explanation could be that these agents prevent the access of 5HT to mainly intraneuronal mono aminooxidases (MAO).
It is well documented that the 5-HT concentrating system of blood platelets is inhibited by drugs in the same way as that of neurons (Todrick & Taite 1969; Paasonen et al. 1971, Sneddon 1973)

Further evidence indicating an effect of DM on 5-HT uptake comes from clinical and pre-clinical interaction studies.

Nierenburg and Semprebon (1993) reported one case of serotonin syndrome precipitated by the concomitant use of phenelzine and DM; fatalities have also been described in patients following the administration of DM and phenelzine (Rivers & Horner 1970; Shamsie & Barriga 1971).
Sinclair (1973) studied the same phenomenon in rabbits: DM administered in rabbits pretreated with phenelzine or nialamide produced symptoms of motor restlessness, tremor, extreme hyperpyrexia and death that are virtually identical to the symptoms associated with human intoxication.

1.5 Dextromethorphan and calcium channels

DM has been widely reported as an NMDA antagonist. In fact, many papers report this drug and its main metabolite, dextrorphan, to exert a protective action in models of hypoxia-ischemia in animals (Prince et al. 1988), NMDA-mediate neurotoxicity (Choi et al. 1987), and NMDA- induced epileptiform activity in brain slices (Apland et al. 1990).
All these actions are exerted in the low micromolar range, i.e. at concentrations far higher than those necessary for the activation of DM high affinity binding site (Kd 22nM).
Dizocilpine, a non-competitive blocker of the NMDA receptor channel acting at the PCP site inside the ion channel competes

only weakly with DM binding (Ki 11.8 μM in Klein et al. 1989). In comparison, DM inhibits the binding of dizocilpine with a Ki of 962 nM (Jaffe et al. 1989). These results suggest an action of DM in the NMDA channel at a site with low affinity for DM and probably belonging to the low affinity binding sites class identified by Klein et al., (1992).

Church et al. (1990) reported the ability of DM to reduce both K^+- and NMDA-evoked increases in $[Ca^{2+}]_i$ in cultured rat hippocampal pyramidal neurons in a concentration-dependent manner (5-50 μM). The DM receptor ligand caramiphen (40 mM) reduced K^+-evoked increase in $[Ca^{2+}]_i$ to a greater extent than the NMDA-evoked increase, while the reverse was true for the PCP receptor ligands ketamine (10-40 μM) and DM (10 μM). These results suggest that DM may mediate its anticonvulsant and neuroprotective effects by reducing Ca^{2+} influx through both voltage-activated and NMDA channels, while other DM ligands may show a greater selectivity for the voltage-activated channel.

Dextromethorphan and cytochrome P450

Once DM enters the bloodstream, it is readily metabolized to dextrorphan through the action of hepatic monoxygenases (Schmid et al., 1985).

DM O-demethylation and debrisoquine 4-hydroxylation are catalyzed by

cytochrochrome P2D6 in humans and 2D1 in rat liver microsomial fraction. Cytochromes catalyze the action of monoxygenases providing the activated oxygen for the hydroxylation.

SKF-525-A (proadifen), a classical inhibitor of several hepatic cytochrome P450 monoxygenases (Schenkman et al., 1972) displaced ^{3}HDM, ^{3}H-(+)-3-PPP and ^{3}HDTG binding with very similar affinities, ranging from 2.4 to 3.7 nM (Klein et al., 1991). A computer assisted modelling analysis of these results showed that SKF-525-A binds to the DM_1/σ_1 site with a Kd of 3nM and with a much lower affinity (Kd=200 nM) at the σ_2 site.
Lobeline, another debrisoquine 4-hydroxylase inhibitor exhibited the same selectivity, with a low affinity at the DM_2 site and high affinity at the DM_1/σ_1 and σ_2 site (Klein et al., 1991).
Other substrates and ligands of debrisoquine-4-hydroxylase such as sparteine, debrisoquine and GBR-12909 were found by several authors to be effective in displacing the σ site ligands DM, PPP, DTG (Ross et al., 1990 and 1991, Contreras et al., 1990, Fleissner et al.,1991).
Nevertheless, Craviso and Musacchio (1982a) showed that the brain microsomal fraction does not have debrisoquine hydroxylase activity; therefore the DM_1/σ_1 site seems not to be,

in all likelihood, debrisoquine hydroxylase. This results do not exclude that this site may represent a different isozyme or a protein sharing homologous sequences with this family of enzymes.

2 Aim of the study

The study of the binding properties of dextromethorphan to guinea pig and rat brain has proven to be a particularly confusing issue. Dextromethorphan binds to two high affinity and two low affinity binding sites (Klein and Musacchio, 1992), and each site has pharmacological properties that overlap with those of a variety of often pharmacologically unrelated ligands (Craviso et al. 1982b).

The lack of selectivity of currently used σ ligands and most of the drugs affecting dextromethorphan binding (e.g. phenothiazines, anticonvulsants) has not permitted the attribution of the pharmacological effect of DM with an action at a specific binding site.

This particular study is aimed at the characterization of distinct dextromethorphan binding sites in rat and human brain on the basis of their differential localization and/or response to ions or drug that have been reported to modulate dextromethorphan binding.

This target can be more easily achieved by the means of autoradiography. In fact this technique allows a straightforward comparison between the levels of expression and characteristics of binding sites for labelled drugs in different brain areas.

A receptor distribution study was firstly pursued, followed by characterization of the pharmacology of dextromethorphan binding to different brain areas.

3.0 Methods

3.1 Sample preparation

3.1.1 DM - Rat brain

Rats were stunned and decapitated, their brain quickly removed and snap-frozen in liquid nitrogen-cooled isopentane. Brain were eventually transferred in a -80° freezer and stored undefinetely. Prior to sectioning, brains were fixed to a cryostat chuck with O.C.T. compund (Tissue-Tek). 10 µm whole brain sagittal sections were cut at -16° at the level of table 78 of Paxinos & Watson`s atlas of rat brain (second edition, academic press) and thaw-mounted on gelatine coated (5% gelatine, 0.05% chromic potassium sulphate) glass slides.

Slide mounted sections were then stored at -20° in sealed plastic bags for not more than one month before being incubated.

3.1.2 DM - Human brain

Human samples were obtained already frozen in liquid nitrogen after a 24-48 hr postmortem delay. Tissue was stored undefinitely

at -80 °. Slabs 2-3 cm thick were obtained cutting the tissue previously equilibrated at -20 ° overnight with a mechanical slicer. Slabs were then flattened up by gentle pressure between two aluminium plates in dry ice and embedded in 2% carboxy methyl cellulose (high viscosity) and stored at -80 °. Prior to sectioning the embedded slabs were left overnight at -20° and then transferred in the cryostat chamber set at -16°.
40 μm sections were cut on Hybond N DNA transfer membrane (Amersham), dried under a stream of cool air for 5-15′, sealed in plastic bags and stored at -20° for not more than 1 week.

3.2 Autoradiography

3.2.1 Preliminary studies

3.2.1.1 DM - Rat brain

On the morning of the experiment, slides were taken off the freezer and left to equilibrate at room temperature for 20 min under a stream of cool air. Slides were then pre-incubated in trisHCl 50 mM pH 7.4 at room temperature for 30 min and dried again as before. After the pre-incubation step, sections were

incubated with 100µl of ^{3}HDM 10 nM in tris 50 mM pH 7.4 at room temperature or at 4°C on a cooling plate. At the end of the incubation, the solution covering the sections was aspirated and the slides rinsed in ice-cooled tris buffer for approximately 3 s and washed for the desired lenght of time in incubation buffer containing choline chloride 0.1 mM and triton X-100 0.01% v/v at 4°C.

The washing troughs contained 300 mL of ice-cooled buffer, and not more than 24 slides were washed in the same bath. After the washing, slides were quickly dipped in distilled water and placed under a fan to dry.

Total and non-specific incubation solutions were made up from a common "hot" solution of ^{3}HDM 13.33 nM at which could be added either 1:4 of cold DM 400 µM or 1:4 of buffer.

Dried slides were eventually cut in pieces, placed in a plastic 20ml scintillation vial and 10 ml "Eco-scint" scintillation fluid added. Vials were then vortexed and counted in a β-scintillation counter.

3.2.1.2 DM -Human brain

Human slide-mounted sections were processed exactly as the rat brain in 3.2.1.1.

Total and non-specific incubation solutions were made up from

a common "hot" solution of ^{3}HDM (1.33 nM) at which could be added either 1:4 of cold DM 400 µM or 1:4 of buffer. Therefore the final concentration of radiolabelled DM in the incubation buffer was 1 nM: this concentration was selected as the lowest concentration giving an adequate number of counts on the basis of preliminary studies.

3.2.1.3 Paroxetine - Rat brain

Experimental conditions for the binding of ^{3}H-paroxetine to rat brain were adapted from the work of Chen et al. (1992). Sections were pre-incubated in tris buffer 50 mM pH 7.4 at room temperature for 20 min. After being dried, rat tissue was incubated with 1 nM ^{3}H-paroxetine in trisHCl 50 mM pH 7.4 containing 300 mM NaCl for 90 min. The solution was eventually aspirated off the sections that were then quickly dipped in buffer and then transferred in a washing trough containing 300 ml of tris 200 mM NaCl. After 30 min of washing, the sections were rinsed in deionized water and placed under a fan to dry.

3.2.2 Autoradiography assay

Sections were processed as for the preliminary study, but following the incubation, they were left to dry overnight.

Subsequently, sections were stuck to cardboard with double-sided adhesive tape, placed in metal autoradiography cassettes and exposed to tritium-sensitive film (^{3}H-Hyperfilm, Amersham) at room temperature for 3-6 weeks.
Tritium standards (Microscales, Amersham) were included in each cassette to allow quantitation. Films were analyzed by the means of a Quantimet 970 (Cambridge Instruments) image analyzer.

4 Results

4.1 Rat Brain

4.1.1 Preliminary studies

4.1.1.1 Binding kinetic: incubation and washing curves

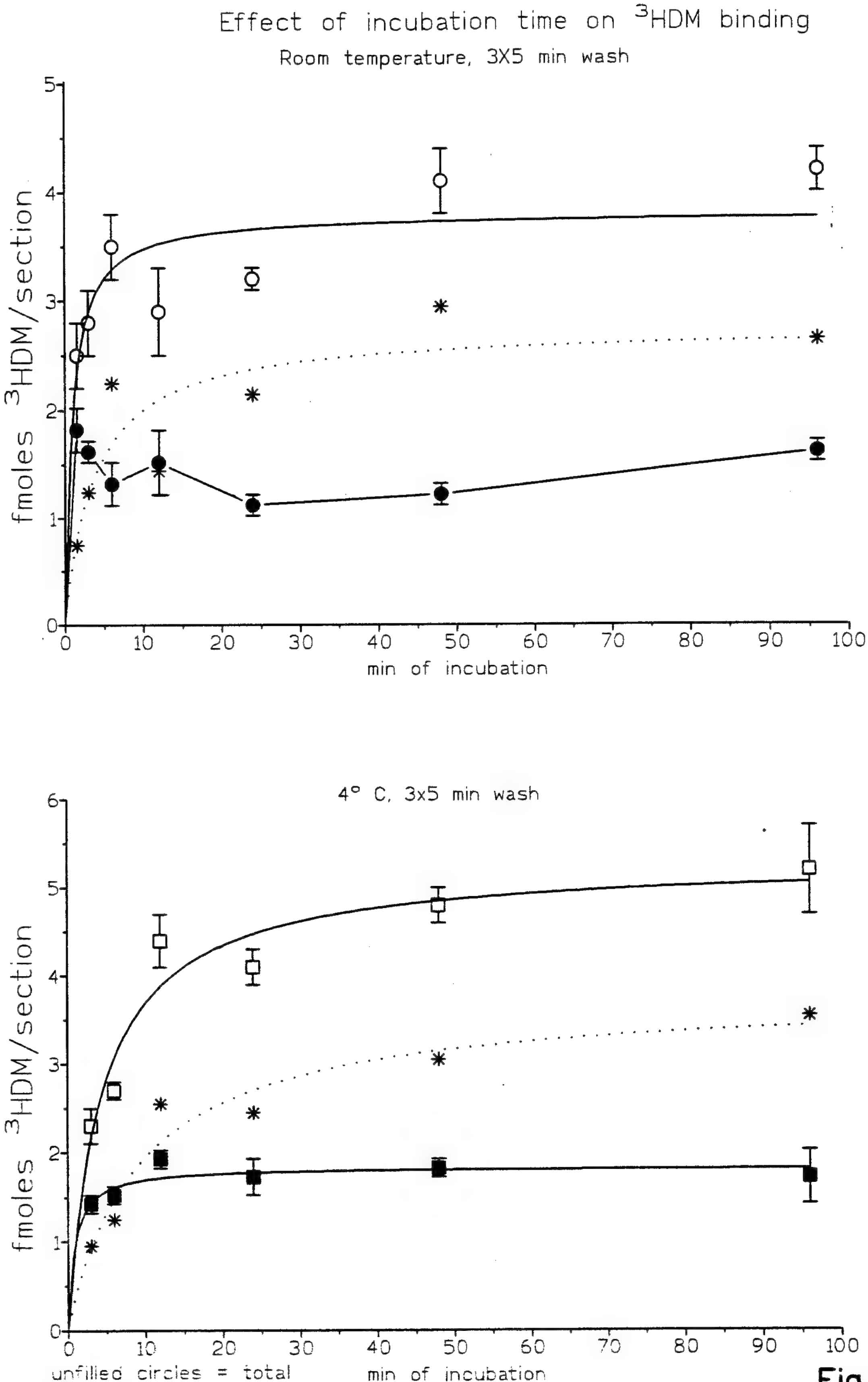
The effect of different incubation times on ^{3}HDM binding in tris 50 mM pH 7.4 was examined at room temperature and at 4° C. Binding reached equilibrium more slowly at 4° than at room temperature, but the level of binding proved to be higher at the lower temperature (fig. 1).

A steady state was considered to be reached after 30′ at room temperature and 60′ at 4°.

Since the levels of binding were higher at 4°, with less variability probably due to a minor effect of evaporation and external temperature, an incubation time of 1h at 4° was selected for all subsequent studies.

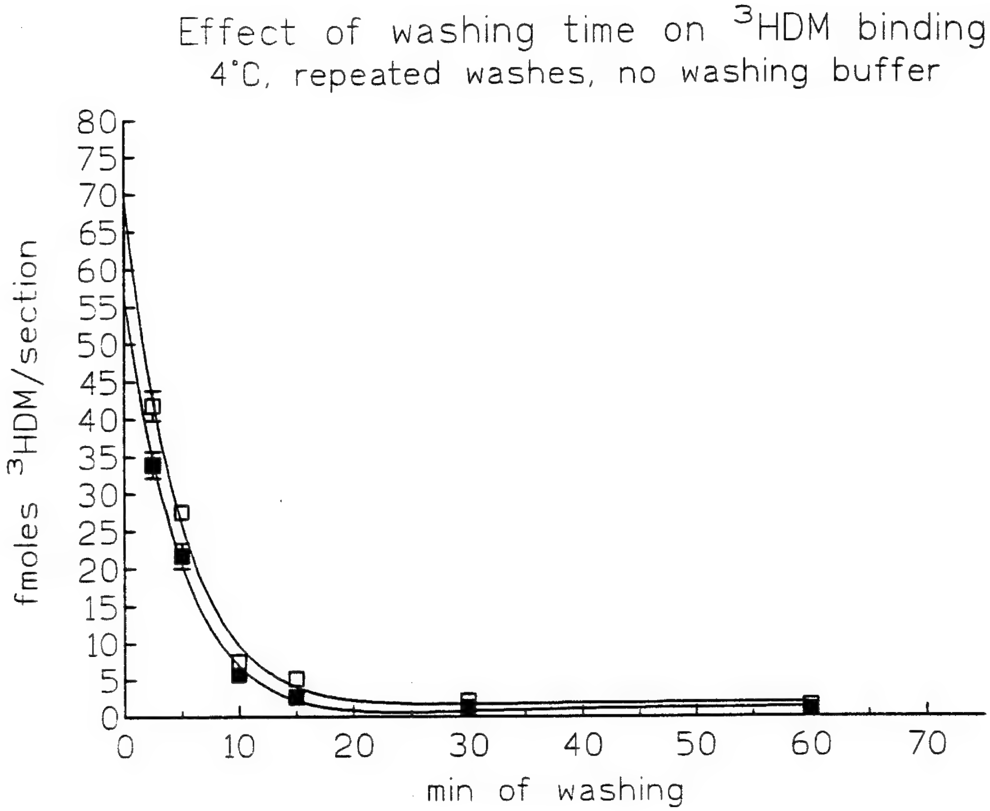
The effect of different washing times is illustrated in fig 2 : slides were washed in incubation buffer which was renewed at 5, 10, 15 and 30 min.

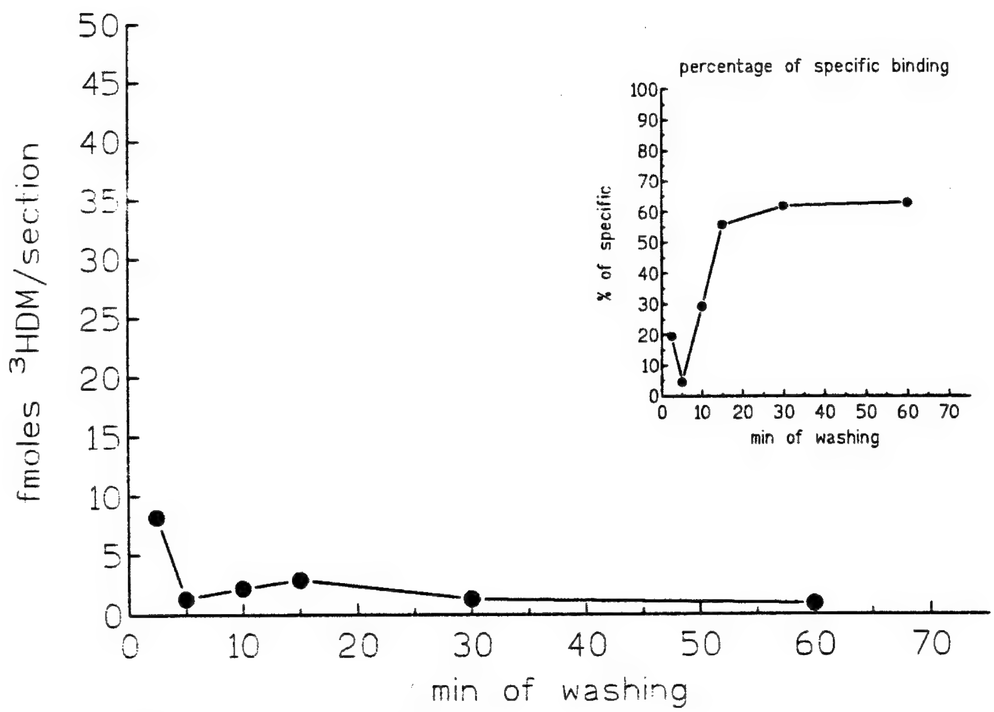
Levels of specific binding were little changed while there was a dramatic decrease in non-specific binding between 2.5 and 15′ of washing. Percentage of specific binding exceeded 50% of total



unfilled circles = total
filled circles = nonspecific
stars = specific

Fig. 1





open squares= total binding
filled squares= non-specific binding
filled circles = specific binding (lower graph)

Fig. 2

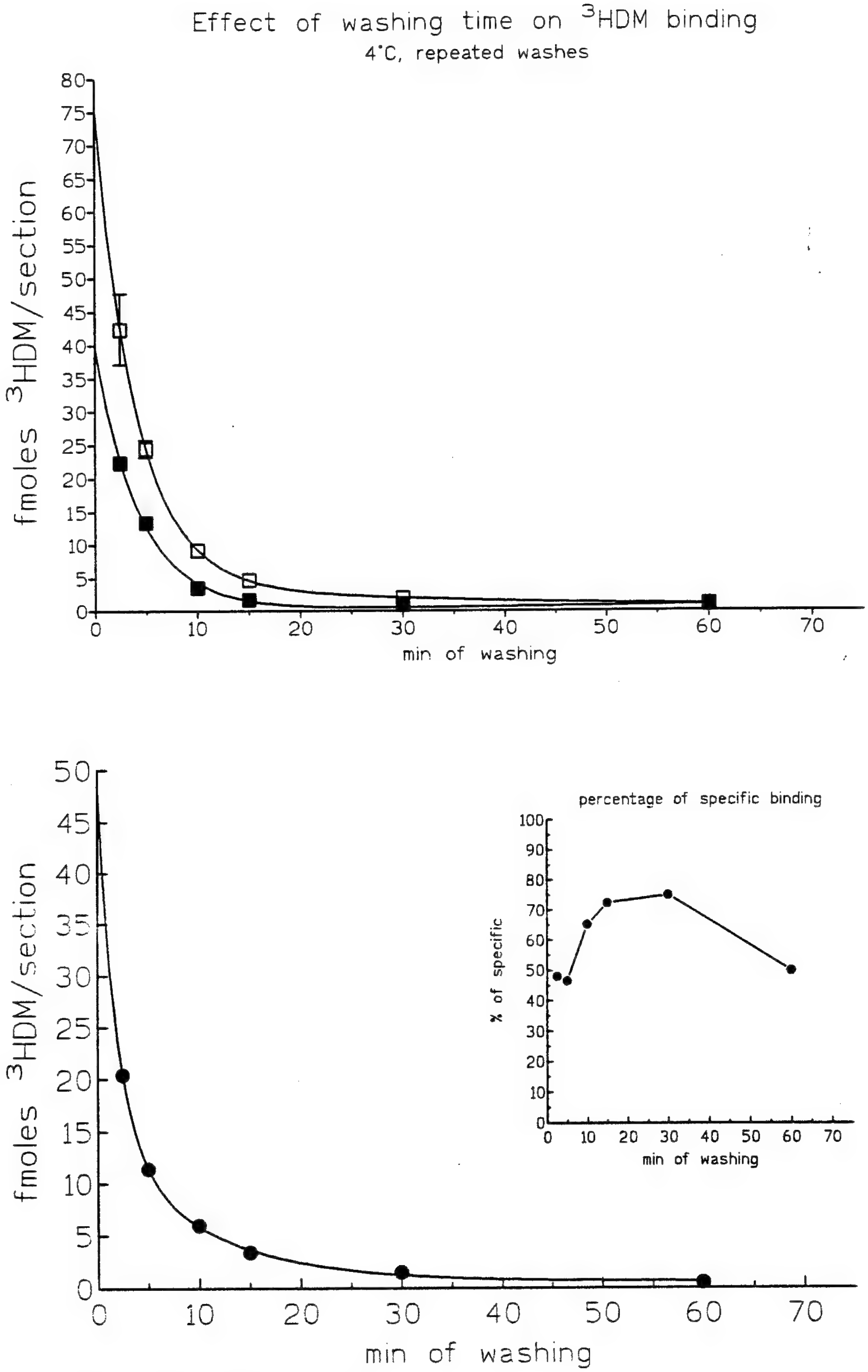
after 15′ of washing.

4.1.1.2 Effect of different washing buffers

Craviso & Musacchio (1982a) reported for DM binding that the most effective washing buffer contained 100 mM choline chloride and 0.01 triton X-100. Here we studied the effect of this buffer on the washing curve of DM using two different protocols. In the first, the washing buffer was changed after 5, 10,15 and 30′, as described in 4.1.1.1. In the second, slides were left in the same buffer solution for the duration of the wash. A decrease in non-specific binding was evident when the washing buffer was changed (fig. 3), but disappeared when the solution was not changed during the wash (fig. 4). Obviously, this effect was more evident at longer washing times.

Interestingly, the levels of non specific binding after 1h washing were considerably higher when the solution was not changed. The process of washing may therefore, be limited by the diffusion of the labelled ligand into the solution.

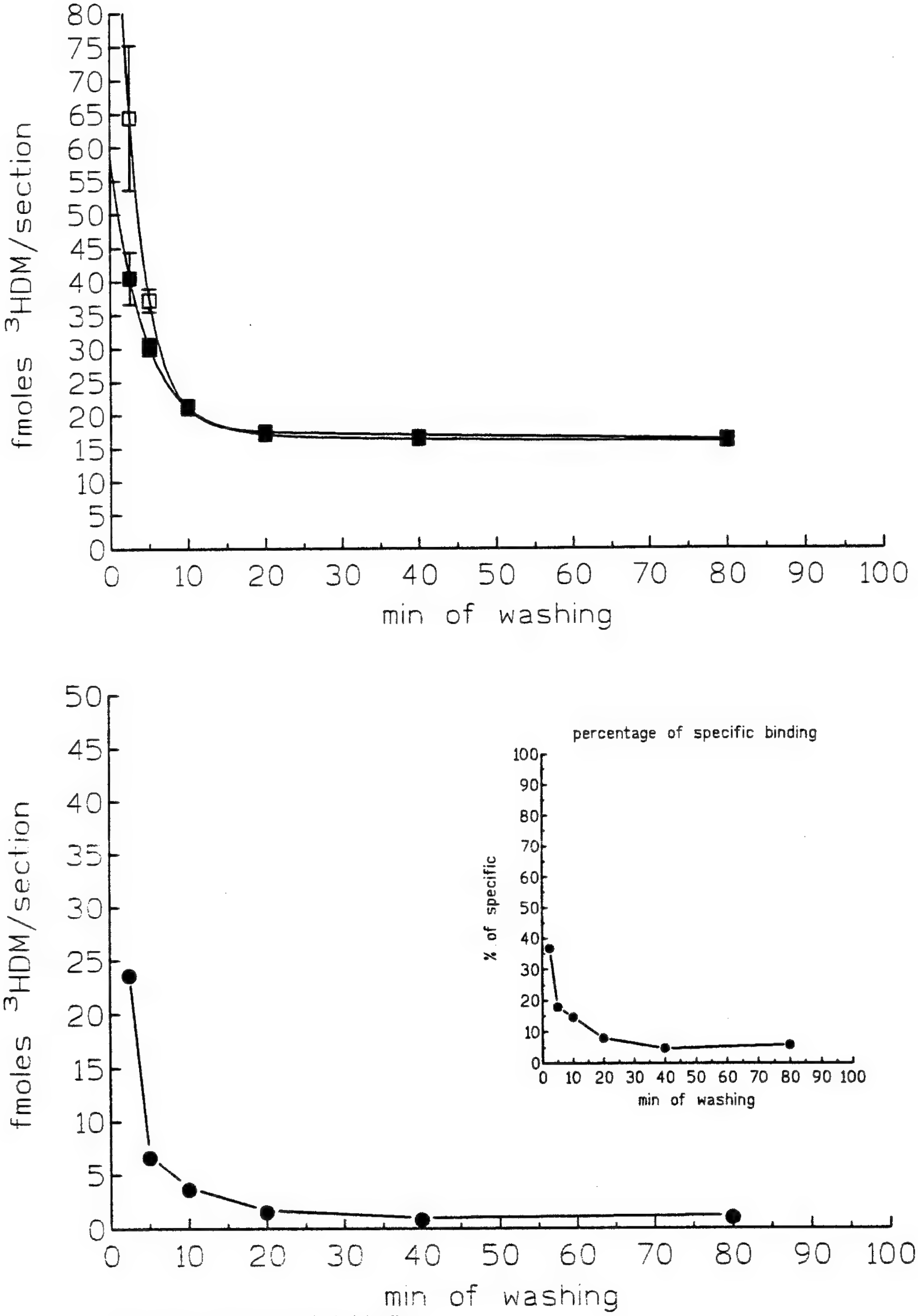
A washing time of 2.5′ with washing buffer was selected for further studies since the higher levels of radiation (and the resulting lower time of exposure for the films) compensated for the lower percentage of specific binding compared to longer washing times.



open squares= total binding
filled squares= non-specific binding
filled circles = specific binding (lower graph)

Fig. 3

Effect of washing time on ^{3}HDM binding
4°C, single wash



open squares= total binding
filled squares= non-specific binding
filled circles = specific binding (lower graph)

Fig. 4

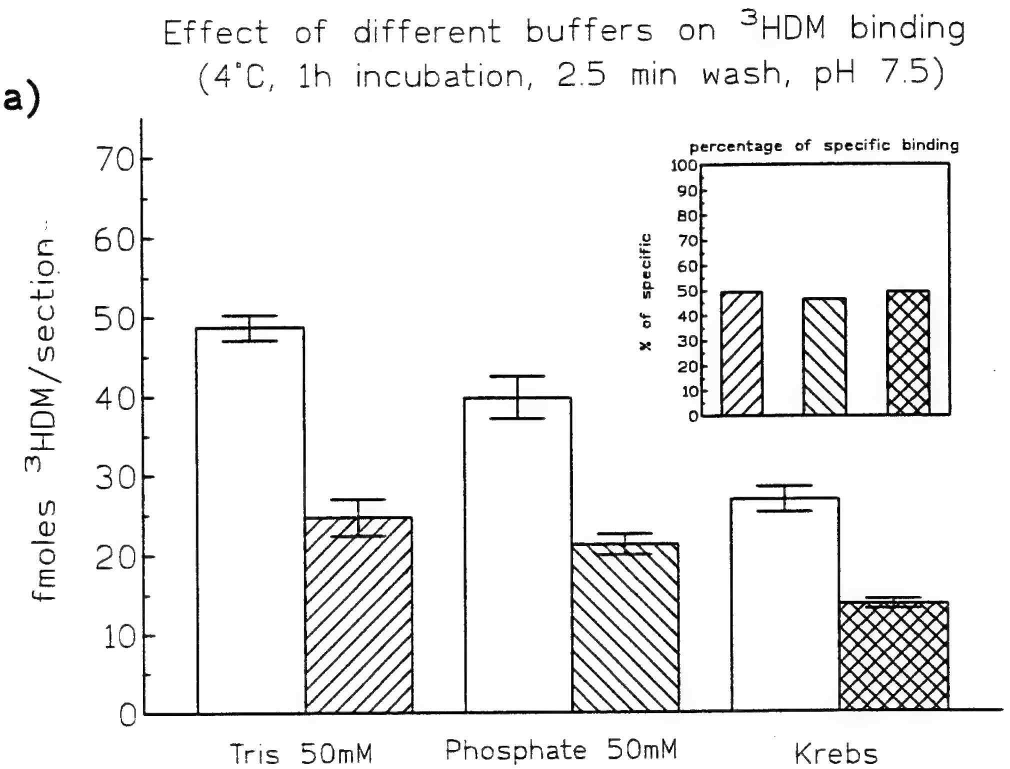
4.1.1.3 Effect of ionic composition on binding

The effect of ionic composition was tested by the means of three different buffers. Levels of DM binding were compared when the incubation buffer was switched from trisHCl to sodium phosphate 50 mM pH 7.4 and Krebs solution. A reduction in both specific and nonspecific binding was noticeable with increased ionic strength. Specific binding remained constant (around 50%) for all the three buffers (fig 5a). TrisHCl 50 mM was therefore selected for subsequent studies.

4.1.1.4 Effect of different pH on binding

The first binding studies for DM were conducted at slightly alkaline pH to increase specific binding (Craviso et al., 1983). The effect of pH ranging from 6.8 to 8.0 was tested in the present work. A selective increase of nonspecific binding was detected at higher pH, with no effect on specific binding (fig. 5b).

a)


Effect of different buffers on ³HDM binding
(4°C, 1h incubation, 2.5 min wash, pH 7.5)
fmoles ³HDM/section
0
10
20
30
40
50
60
70
Tris 50mM
Phosphate 50mM
Krebs
percentage of specific binding
% of specific
100
90
80
70
60
50
40
30
20
10
0

b)

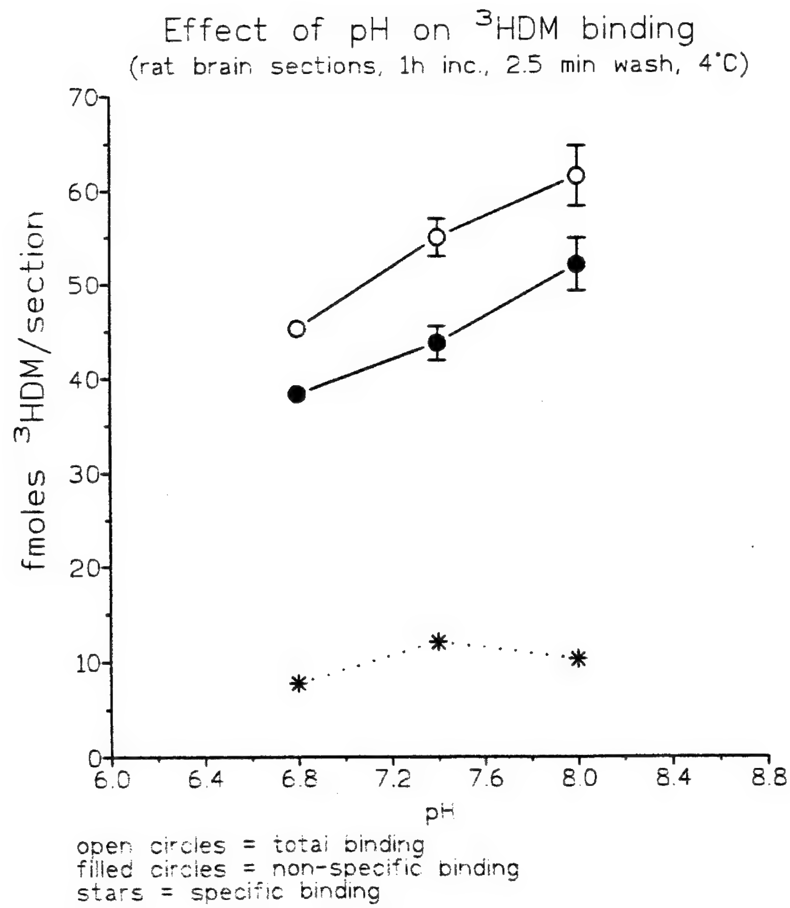

Effect of pH on ³HDM binding
(rat brain sections, 1h inc., 2.5 min wash, 4°C)
fmoles ³HDM/section
0
10
20
30
40
50
60
70
6.0
6.4
6.8
7.2
7.6
8.0
8.4
8.8
pH
open circles = total binding
filled circles = non-specific binding
stars = specific binding

Fig. 5

4.1.2 Autoradiography

4.1.2.1 Effect of Na^+ on DM binding

Sodium (50 and 120 mM) produced a dramatic increase in ^{3}HDM binding in most of the areas of rat brain. DM binding to medial mammillary (MM), dorsal raphe (DR), ventral tegmental area (VTA), medial pretectal nuclei (MPT), anterior hypothalamus (AH), central grey (CG), superior colliculi (SC), dorsal tegmental area (DTG), frontal cortex (FC), mediodorsal thalamic nuclei (MD), pons (P), dentate gyrus (DG), and CA3 field of the hippocampus was significantly increased (fig. 6).
Na^+-dependent binding to the ventral tegmental area was five times higher than control binding in the absence of Na^+.
Binding to occipital cortex (OC) and cerebellum (CER) was not sensitive to sodium.

4.1.2.2 Effect of Calcium and Magnesium on Na-dependent DM binding

Calcium and Magnesium at the physiological concentrations of 2.5 and 1.2 mM respectively both reduced the binding of DM in the presence of sodium 120 mM. The effect was statistically

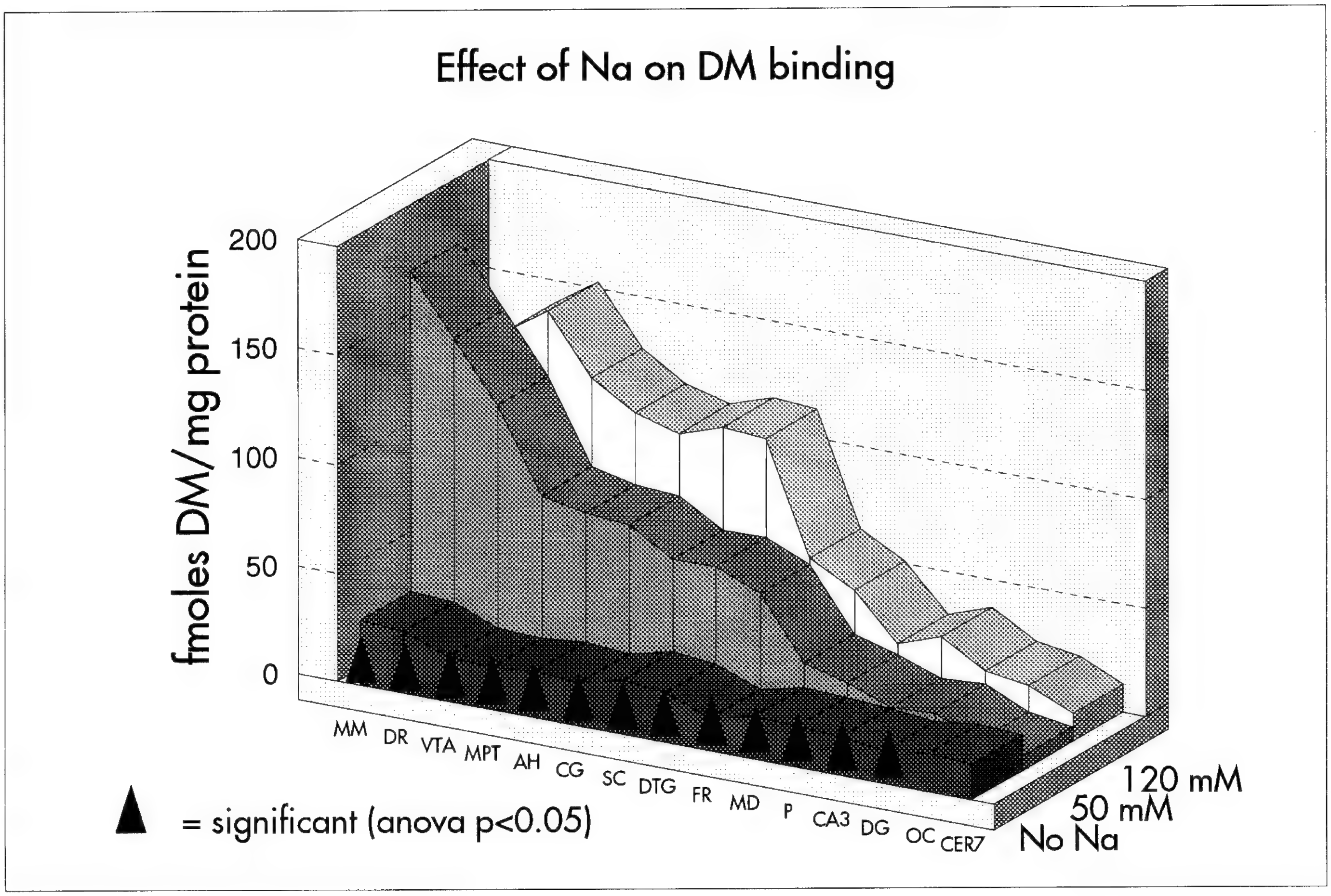
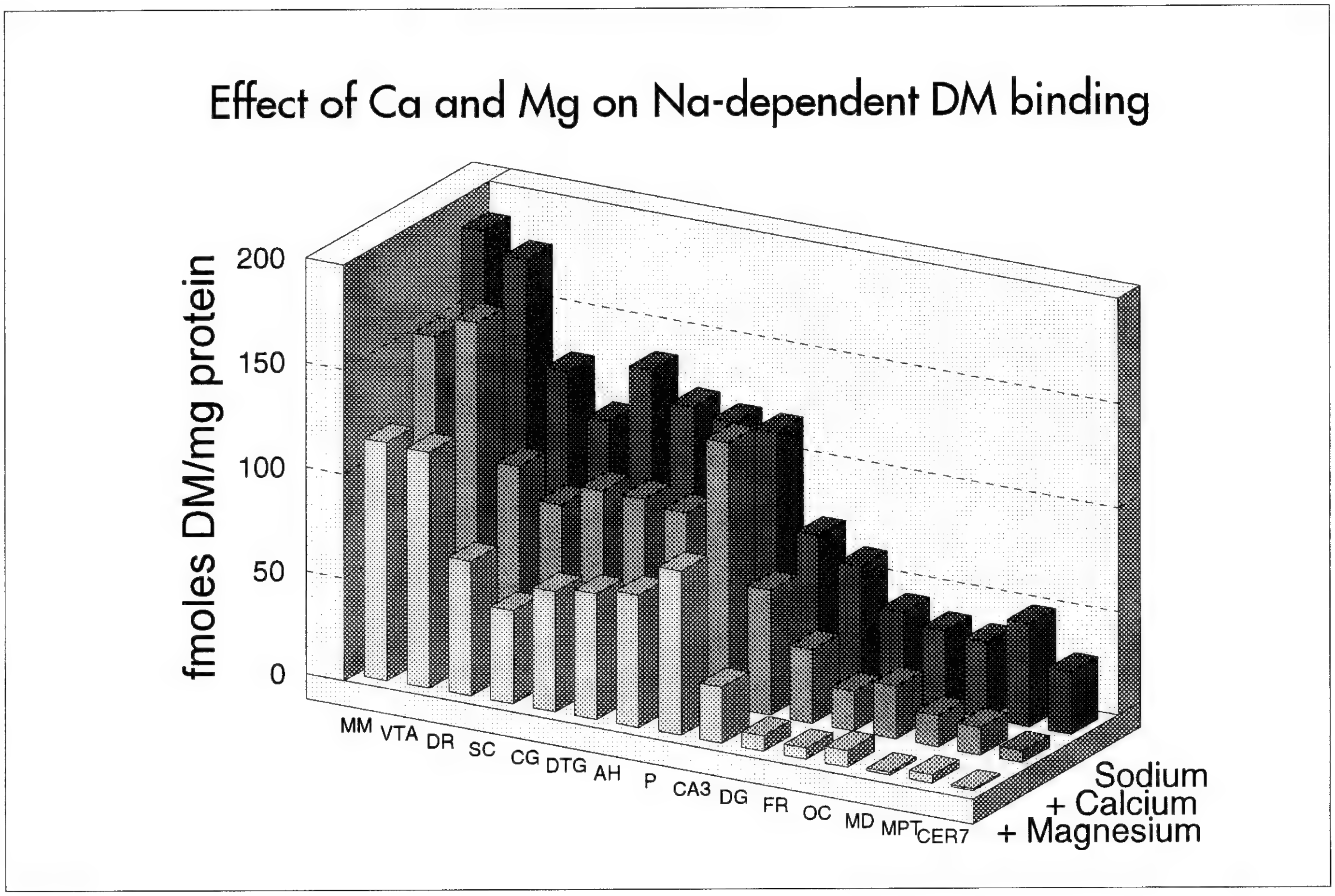

Effect of Na on DM binding
fmoles DM/mg protein
200
150
100
50
0
MM DR VTA MPT AH CG SC DTG FR MD P CA3 DG OC CER7
120 mM
50 mM
No Na
= significant (anova p<0.05)

Fig. 6



Effect of Ca and Mg on Na-dependent DM binding
fmoles DM/mg protein
200
150
100
50
0
MM
VTA
DR
SC
CG
DTG
AH
P
CA3
DG
FR
OC
MD
MPT
CER7
Sodium
+ Calcium
+ Magnesium

Fig. 7

significant in all the nuclei detected, and was slightly larger for magnesium. In the presence of magnesium, DM binding was reduced to 50% of control, while calcium reduced the binding by approximately 30% (fig. 7).

4.1.2.3 Distribution of DM binding to rat brain

In the presence of Na^+ (120 mM), high levels of DM binding (100-150 fmoles/mg protein) were detected in the midbrain, at the level of the VTA, MM, DR, SC, MPT, CG and the DTG. AH, FR and MD showed medium levels of binding (50-100 fmoles/mg protein). Whereas hippocampus (CA3 and DG), P, OC and CER exhibited low levels of DM binding (fig. 8).
In the absence of Na^+, the binding of all regions except OC and CER was reduced to 20-40 fmoles/mg of protein, the overall distribution being much less discrete.

4.1.2.4 ^{3}HDM saturation curves

DM binding to rat brain was measured at concentrations ranging from 5 to 80 nM in the presence of Na^+ 120mM. DM displayed high affinity, saturable binding in all 15 nuclei detected. At the concentration of 10 nM, specific binding reached an average of

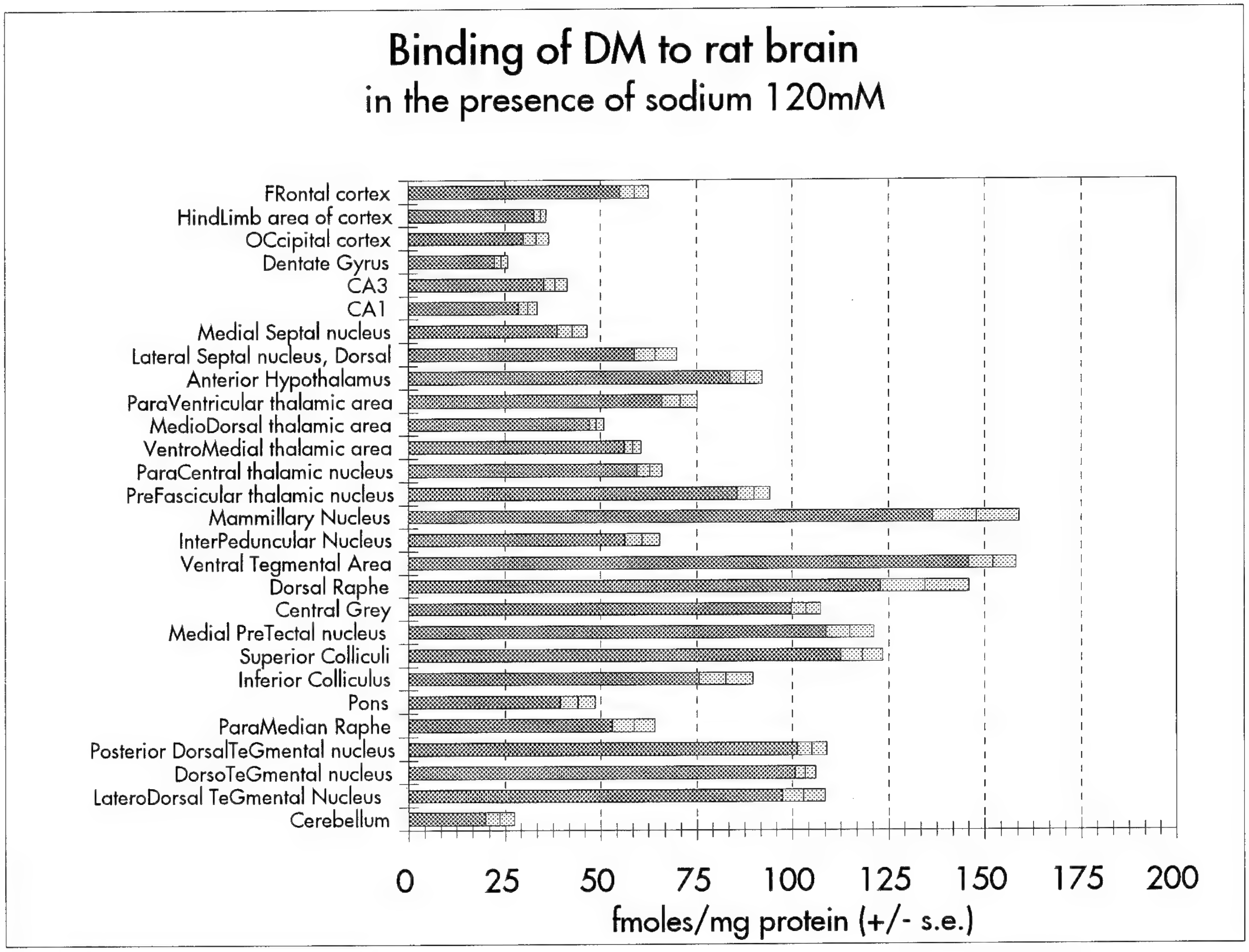
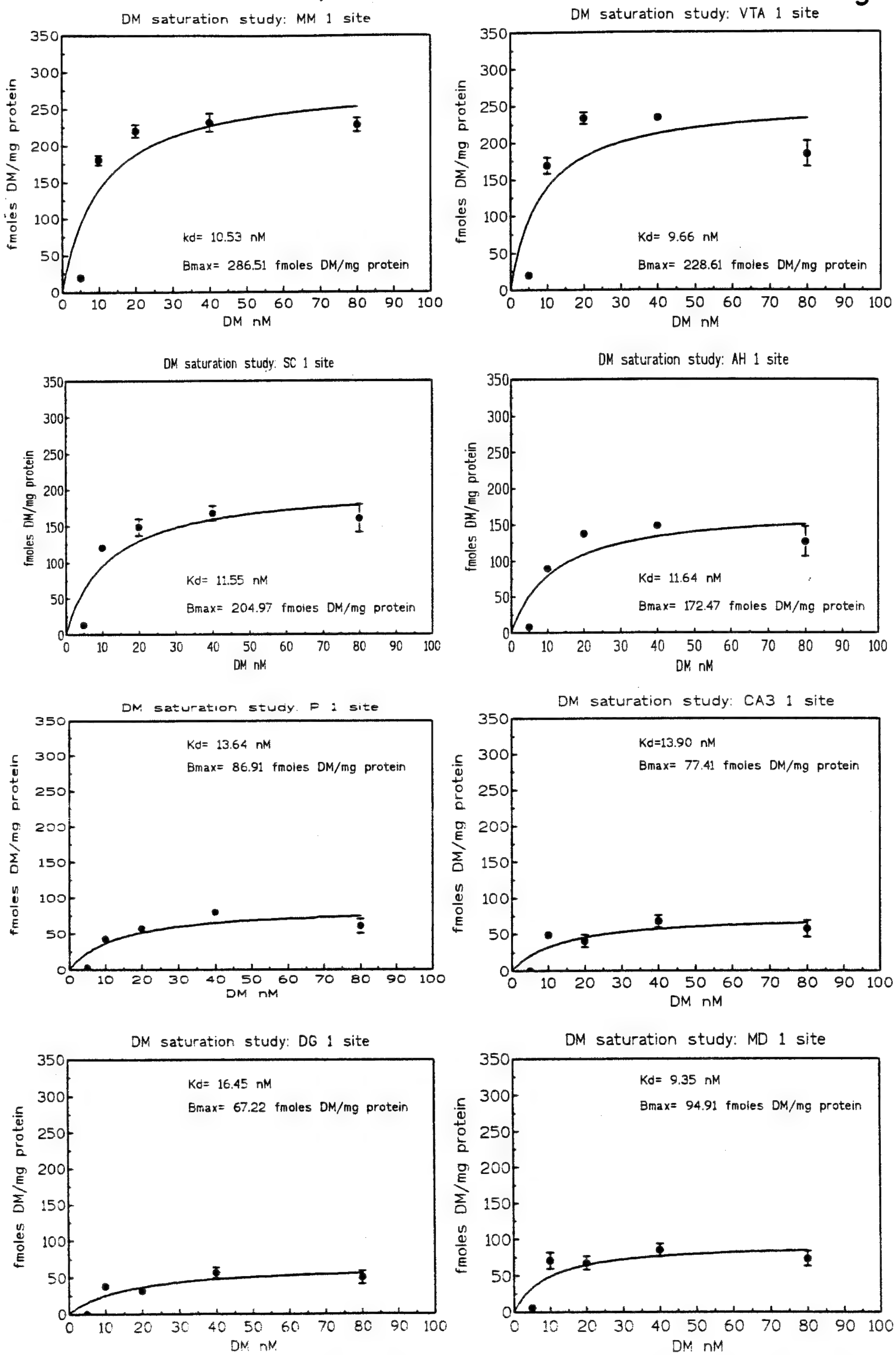

Binding of DM to rat brain
in the presence of sodium 120mM
FRontal cortex
HindLimb area of cortex
OCcipital cortex
Dentate Gyrus
CA3
CA1
Medial Septal nucleus
Lateral Septal nucleus, Dorsal
Anterior Hypothalamus
ParaVentricular thalamic area
MedioDorsal thalamic area
VentroMedial thalamic area
ParaCentral thalamic nucleus
PreFascicular thalamic nucleus
Mammillary Nucleus
InterPeduncular Nucleus
Ventral Tegmental Area
Dorsal Raphe
Central Grey
Medial PreTectal nucleus
Superior Colliculi
Inferior Colliculus
Pons
ParaMedian Raphe
Posterior DorsalTeGmental nucleus
DorsoTeGmental nucleus
LateroDorsal TeGmental Nucleus
Cerebellum
0
25
50
75
100
125
150
175
200
fmoles/mg protein (+/- s.e.)

Fig. 8

Analysis of ^{3}HDM saturation

Fig. 9



66% of total binding, the highest value being in the MM with 83%.

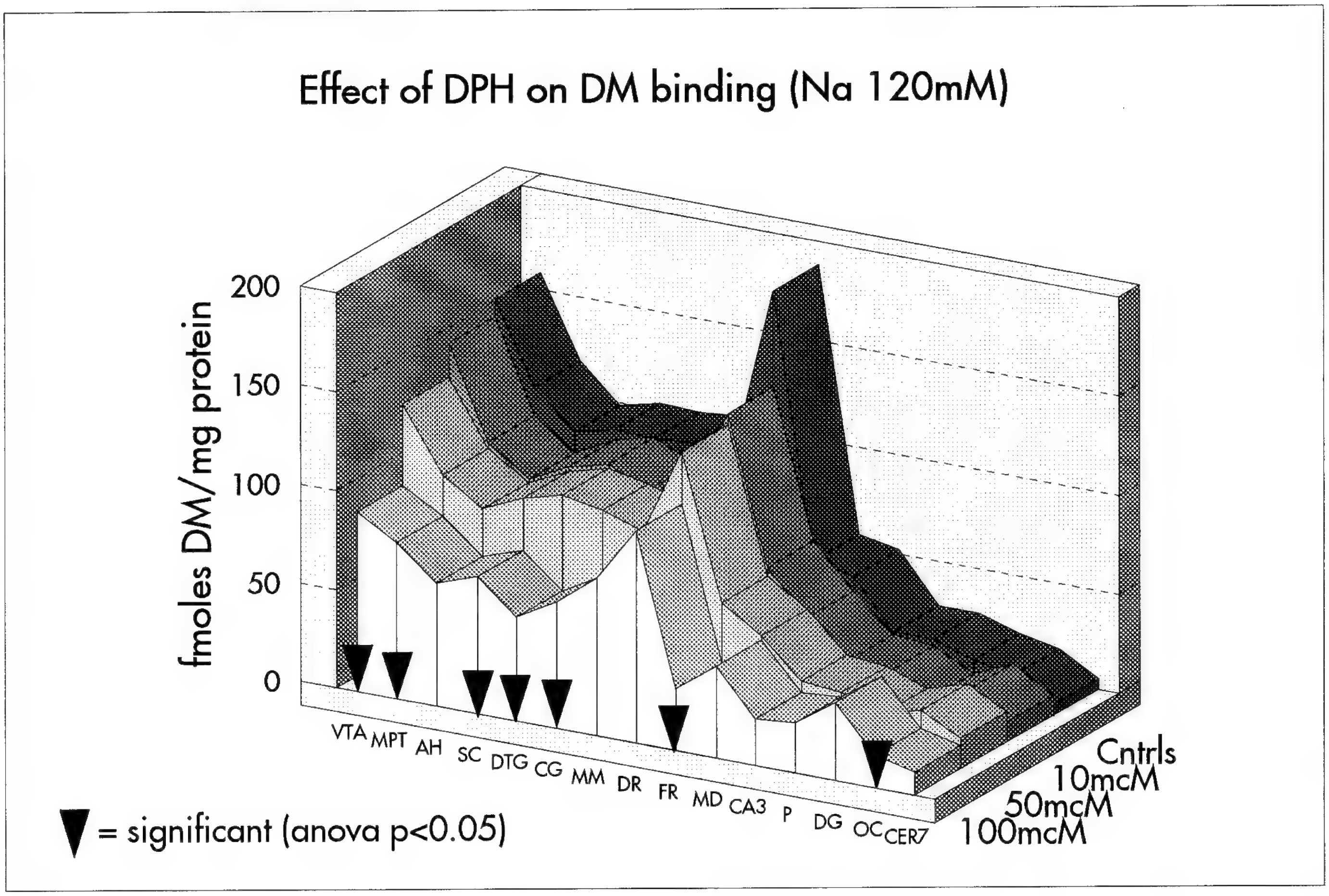
Binding to 8 of the detected nuclei was selected for further analysis (fig. 9), results being shown in fig. 8. Binding data fitted with a one-site model showed affinities ranging from 9.4 nM (MD) to 13.9 nM (hippocampus` CA3 field). A two-site model did not significantly improve the fit in any region.

4.1.2.5 Effect of phenytoin (DPH) on DM binding

DPH was tested on DM binding at concentrations ranging from 10 to 100 μM. In the presence of Na^+ 120 mM, DPH induced a concentration-dependent inhibition of ^{3}HDM binding in most of the measured areas. Binding to VTA, MPT, SC, DTG, CG, FR and OC was significantly reduced. Binding to AH and DR appeared to be lower but was not significantly different from control (fig. 10).

Binding to MD, CA3 field and DG of the hippocampus, P and CER was unaffected.

In the absence of Na^+ (fig. 11), the effect of phenytoin was less evident, and statistical significance was reached only in VTA, DTG, CG, MM and FR.



Effect of DPH on DM binding (Na 120mM)
fmoles DM/mg protein
200
150
100
50
0
VTA
MPT
AH
SC
DTG
CG
MM
DR
FR
MD
CA3
P
DG
OC
CER7
Cntrls
10mcM
50mcM
100mcM
= significant (anova p<0.05)

Fig. 10

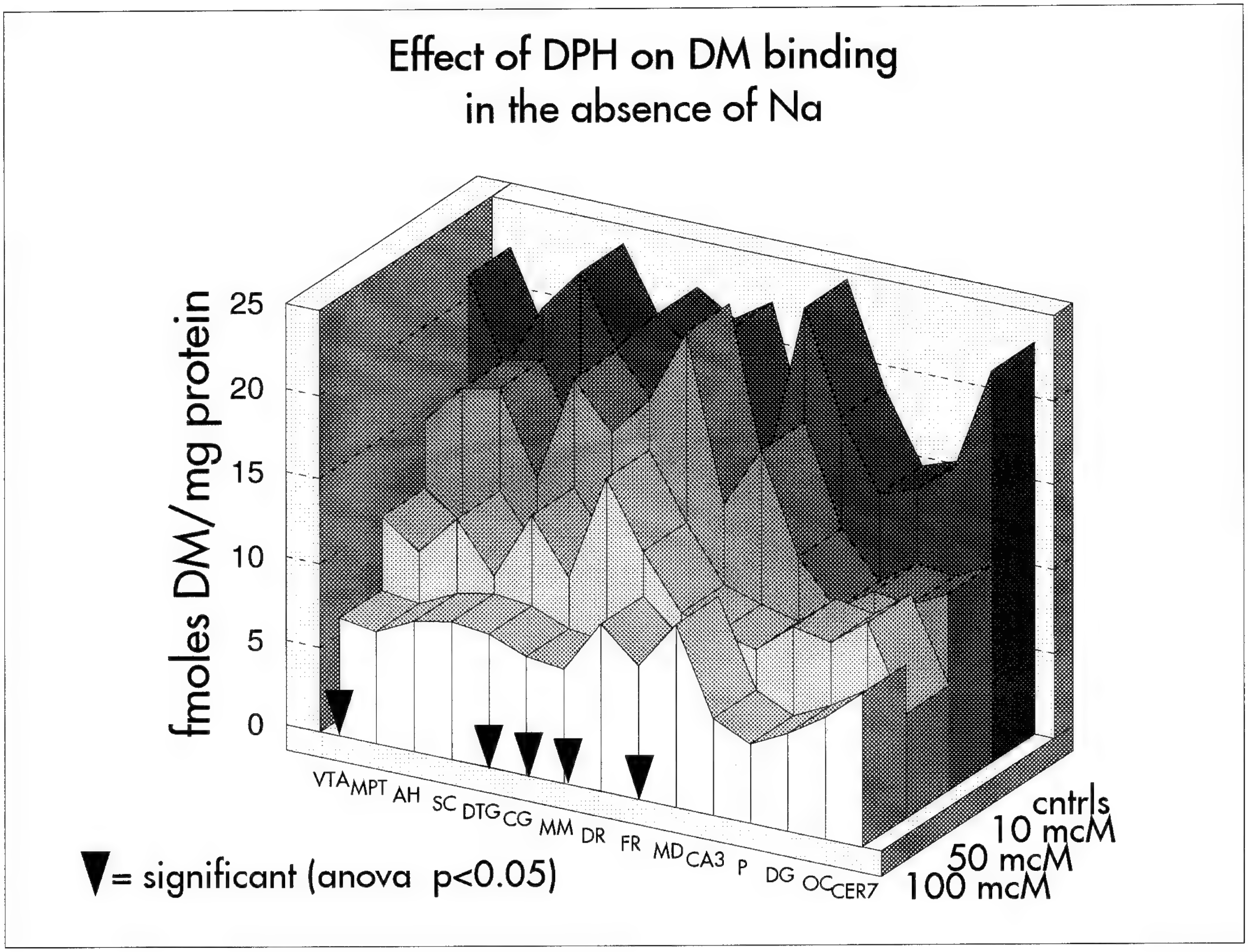

Effect of DPH on DM binding
in the absence of Na
fmoles DM/mg protein
25
20
15
10
5
0
VTA MPT AH SC DTG CG MM DR FR MD CA3 P DG OC CER7
cntrls
10 mcM
50 mcM
100 mcM
▼= significant (anova p<0.05)

Fig. 11

4.1.2.6 Effect of (+)-PPP on DM binding

In the presence of sodium 120 mM, no clear concentration-dependent effect of (+)-PPP could be detected on DM binding to areas with high binding levels such as VTA, MPT, AH, SC, DTG, CG, MM and DR. However, a significant reduction in DM binding was observed in the DG (fig. 12).
In the absence of sodium, PPP at the highest concentration of 176 nM enhanced the binding of DM to all the nuclei except CA3 and CER (fig. 13).

4.1.2.7 Effect of paroxetine (PX) on sodium-dependent DM binding

PX induced a decrease in Na-dependent DM binding that was statistically significant in all nuclei tested. The effect became apparent at 10 nM, and was concentration dependent with a reduction in binding at 100 nM PX of approximately 30% (fig. 14).

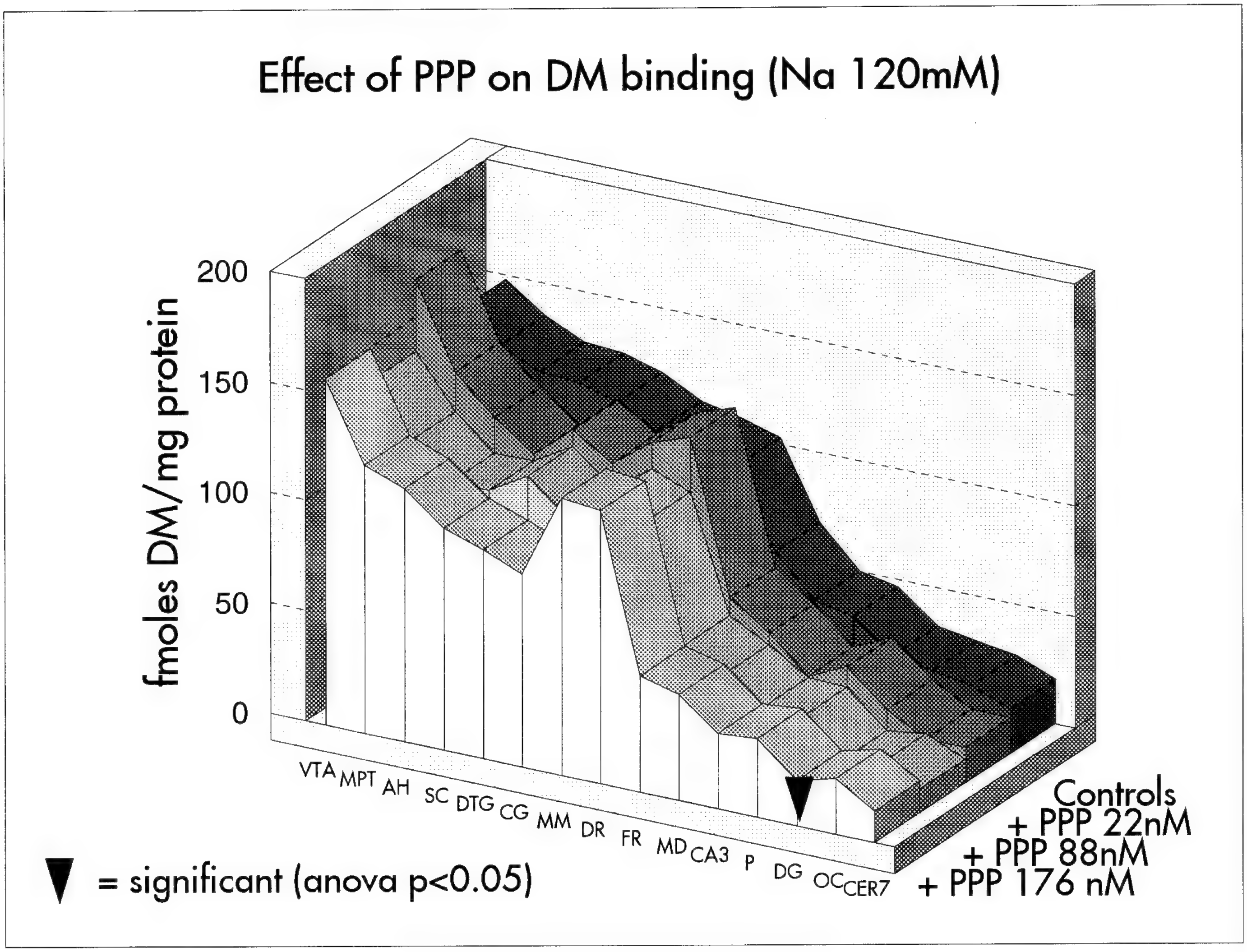

Effect of PPP on DM binding (Na 120mM)
fmoles DM/mg protein
200
150
100
50
0
VTA
MPT
AH
SC
DTG
CG
MM
DR
FR
MD
CA3
P
DG
OC
CER7
Controls
+ PPP 22nM
+ PPP 88nM
+ PPP 176 nM
= significant (anova p<0.05)

Fig. 12

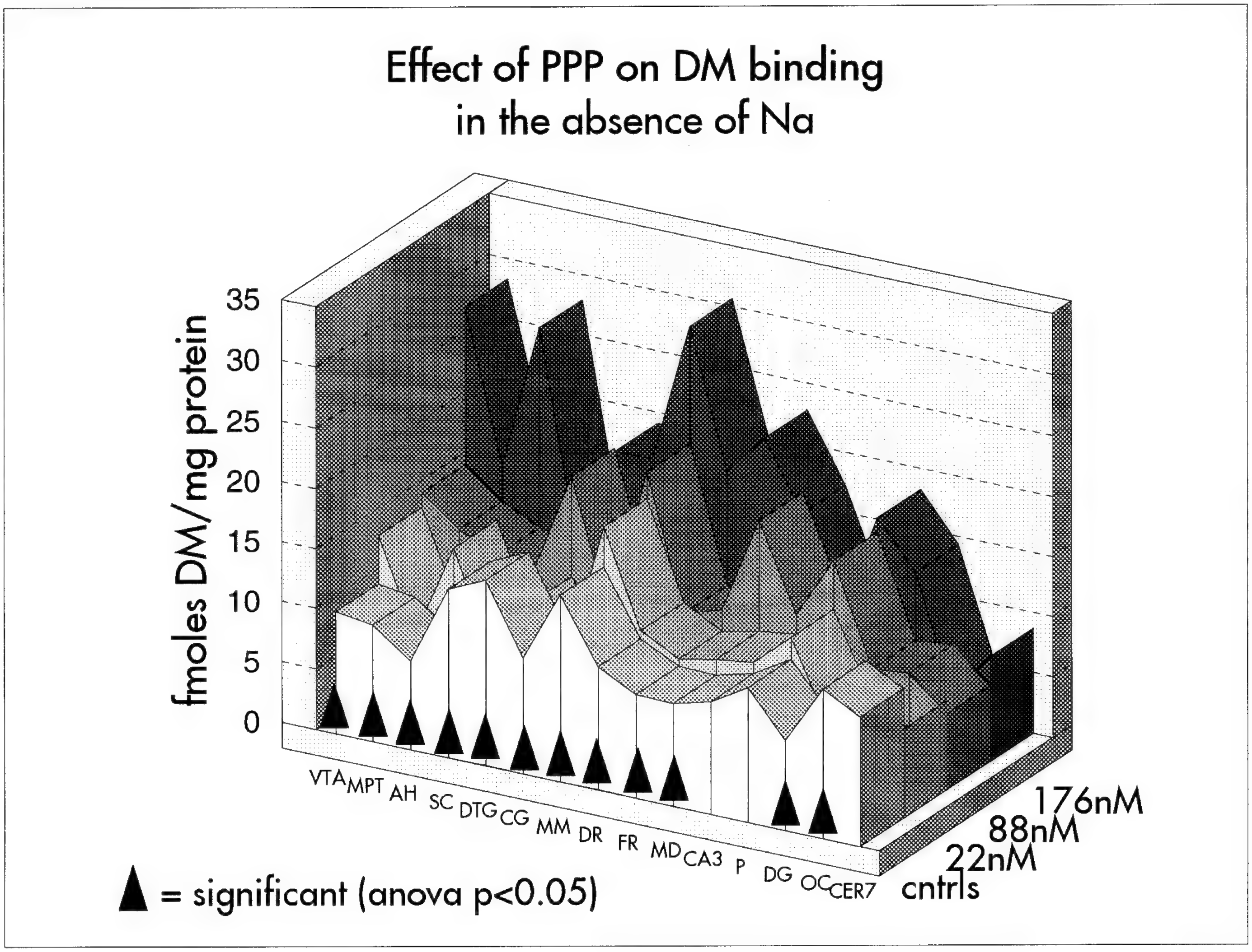

Effect of PPP on DM binding
in the absence of Na
fmoles DM/mg protein
35
30
25
20
15
10
5
0
VTA
MPT
AH
SC
DTG
CG
MM
DR
FR
MD
CA3
P
DG
OC
CER7
176nM
88nM
22nM
cntrls
= significant (anova p<0.05)

Fig. 13

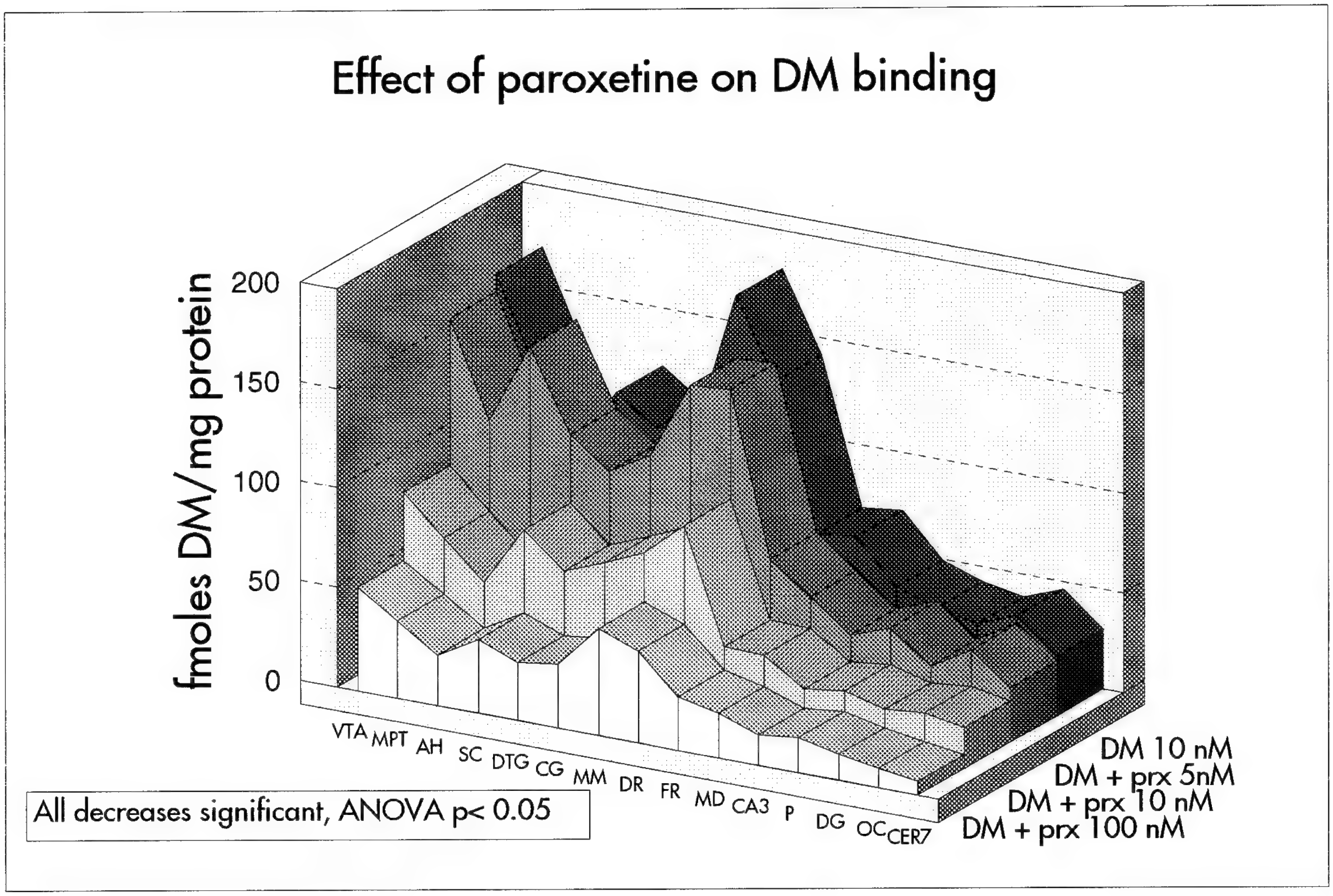

Effect of paroxetine on DM binding
fmoles DM/mg protein
200
150
100
50
0
VTA
MPT
AH
SC
DTG
CG
MM
DR
FR
MD
CA3
P
DG
OC
CER7
DM 10 nM
DM + prx 5nM
DM + prx 10 nM
DM + prx 100 nM
All decreases significant, ANOVA p< 0.05

Fig. 14

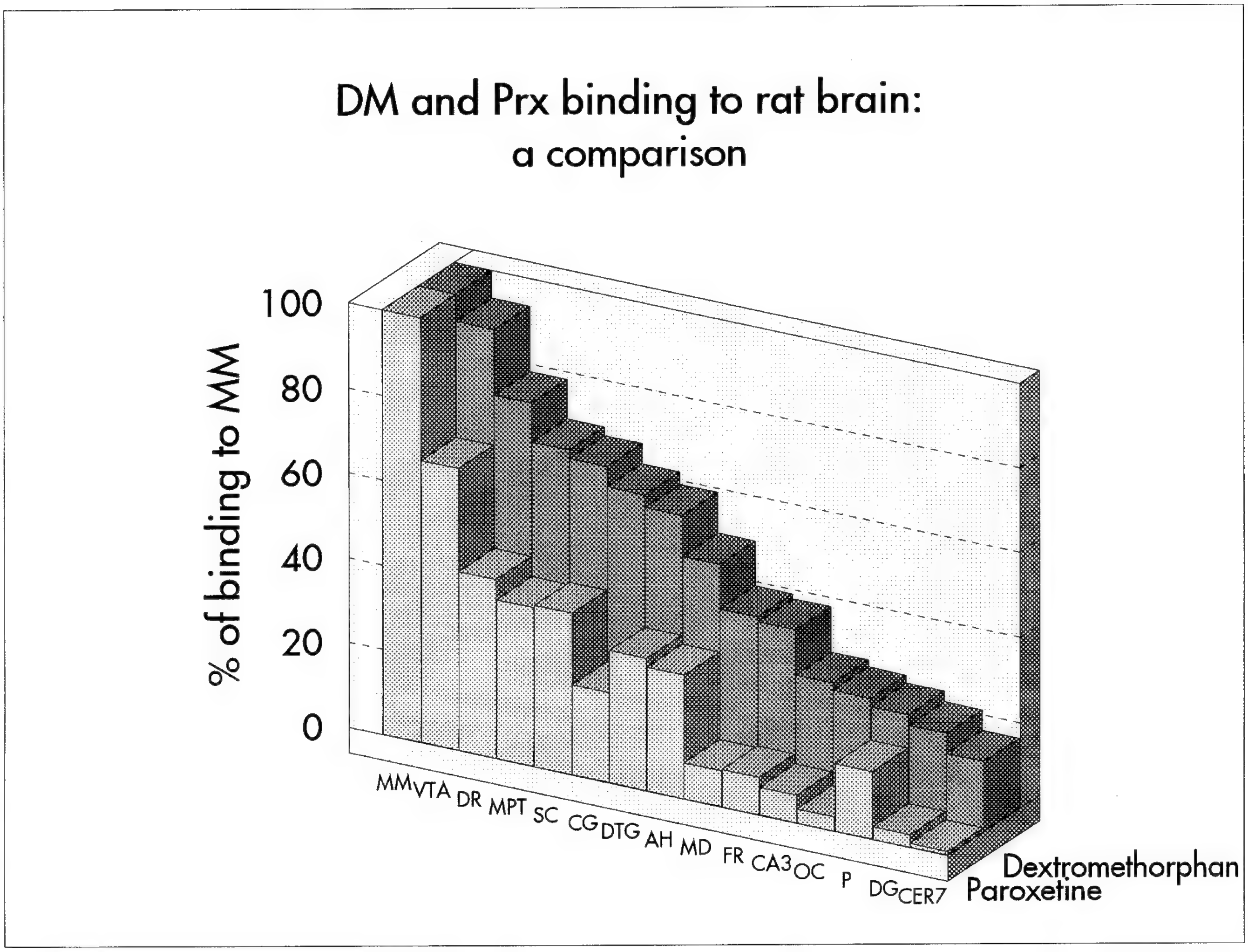
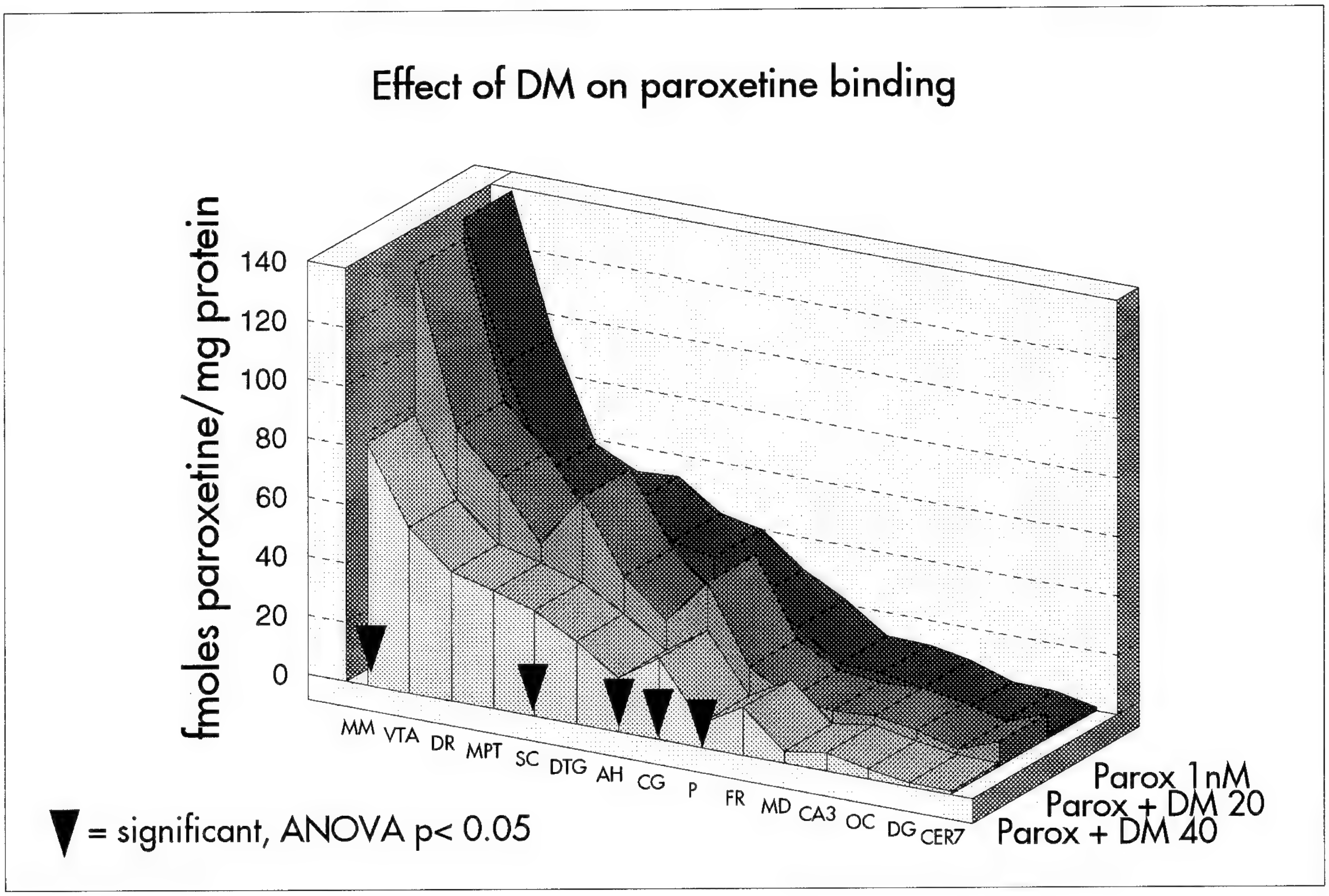

DM and Prx binding to rat brain:
a comparison
% of binding to MM
100
80
60
40
20
0
MM
VTA
DR
MPT
SC
CG
DTG
AH
MD
FR
CA3
OC
P
DG
CER7
Dextromethorphan
Paroxetine

Fig. 15



Effect of DM on paroxetine binding
fmoles paroxetine/mg protein
140
120
100
80
60
40
20
0
MM VTA DR MPT SC DTG AH CG P FR MD CA3 OC DG CER7
Parox 1nM
Parox + DM 20
Parox + DM 40
= significant, ANOVA p< 0.05

Fig. 16

4.1.2.8 Effect of DM on paroxetine binding

The ability of DM to displace paroxetine binding was examined in this part of the study. The distribution of paroxetine binding sites correlated quite well with that of DM (fig. 15). DM at the concentration of 40 nM was effective in displacing paroxetine binding from MM, SC, AH, CG and P. All other areas showed no significant difference from controls (fig. 16).

4.2 Human Brain

4.2.1 Preliminary studies

4.2.1.1 Effect of different buffers on binding

The effect of different buffers was tested on DM binding to human cortex. While the total binding to the section decreased with increasing ionic strength, the percentage of specific binding was optimal with sodium phosphate 50 mM pH 7.4 and Krebs solution (fig.17).

On the basis of these results and of the characteristics of DM binding to rat brain, sodium phosphate was selected for further studies.

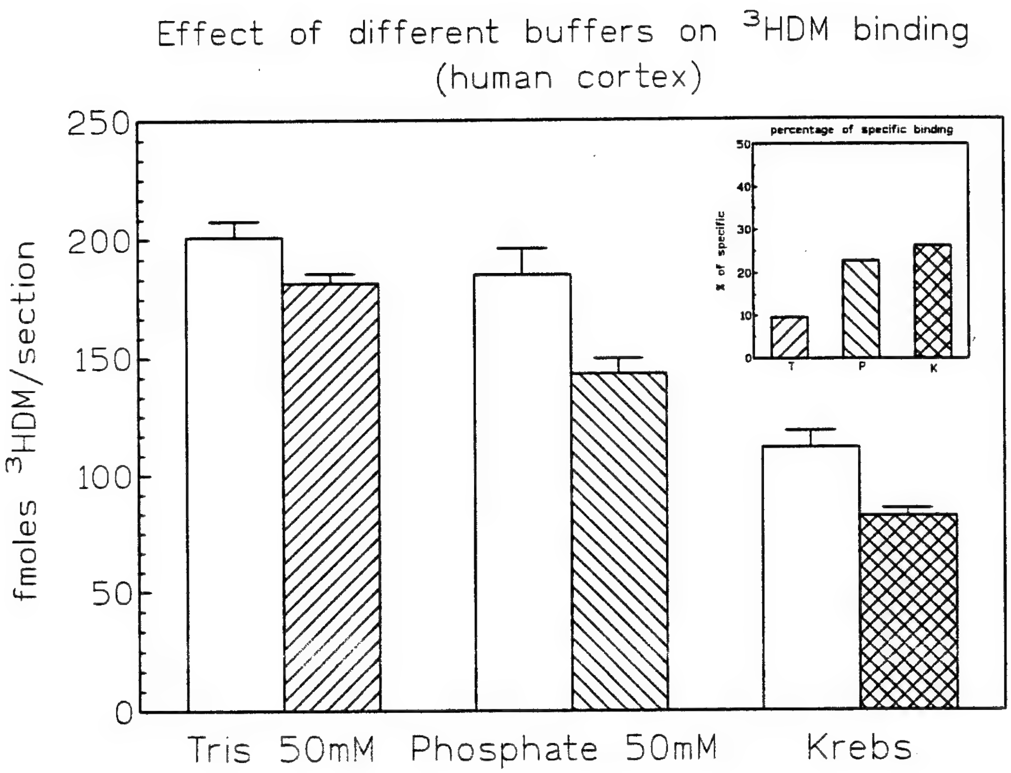
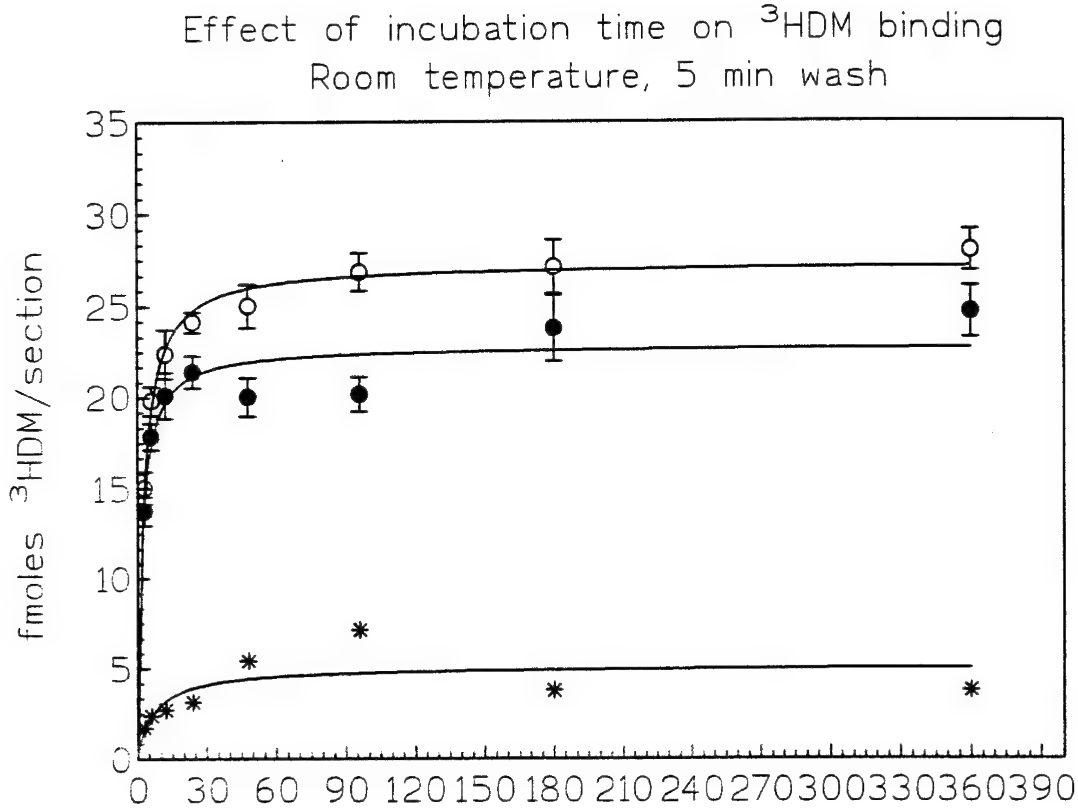
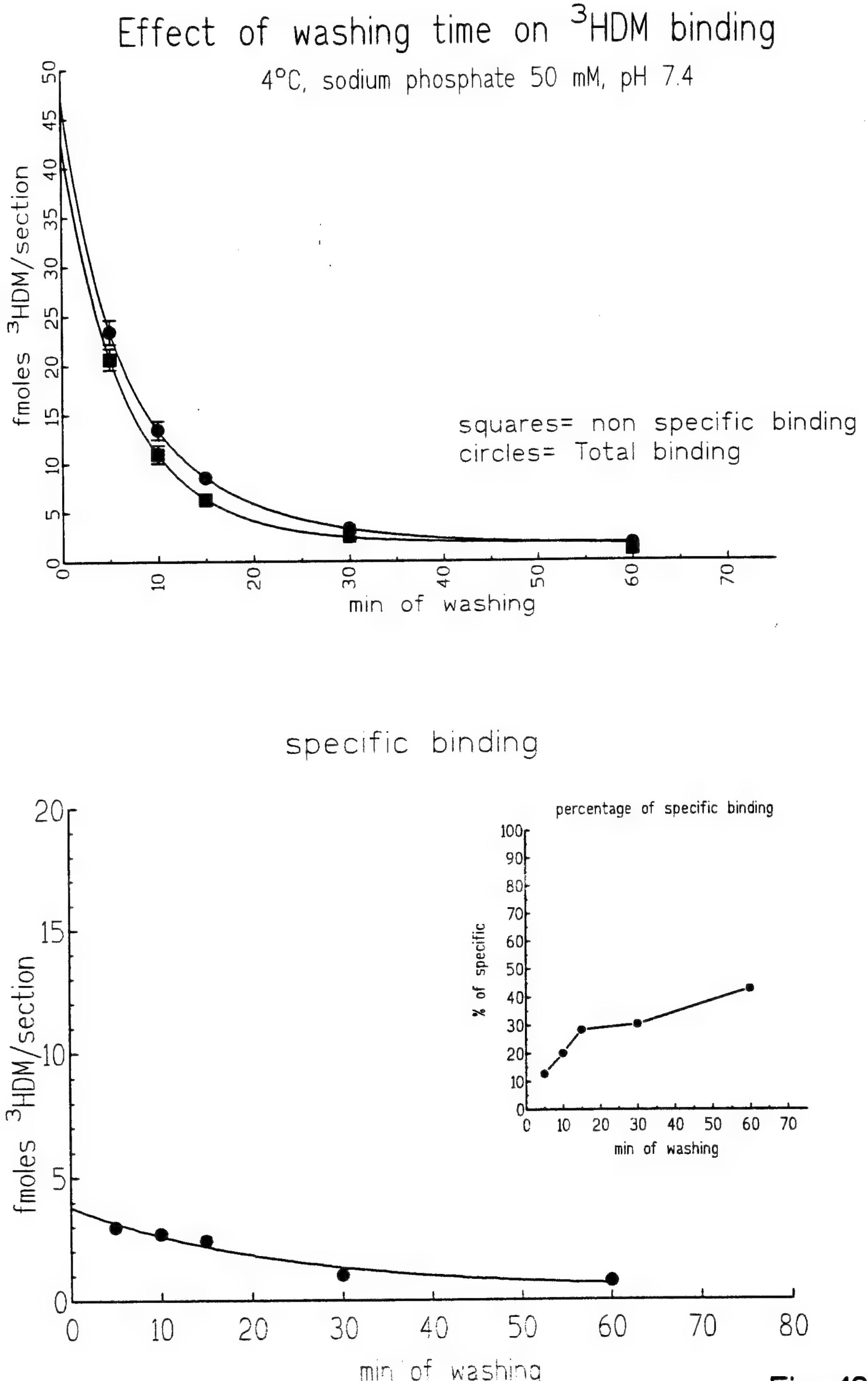

Effect of different buffers on ³HDM binding
(human cortex)
fmoles ³HDM/section
250
200
150
100
50
0
Tris 50mM
Phosphate 50mM
Krebs
percentage of specific binding
% of specific
50
40
30
20
10
0
T
P
K

Fig. 17



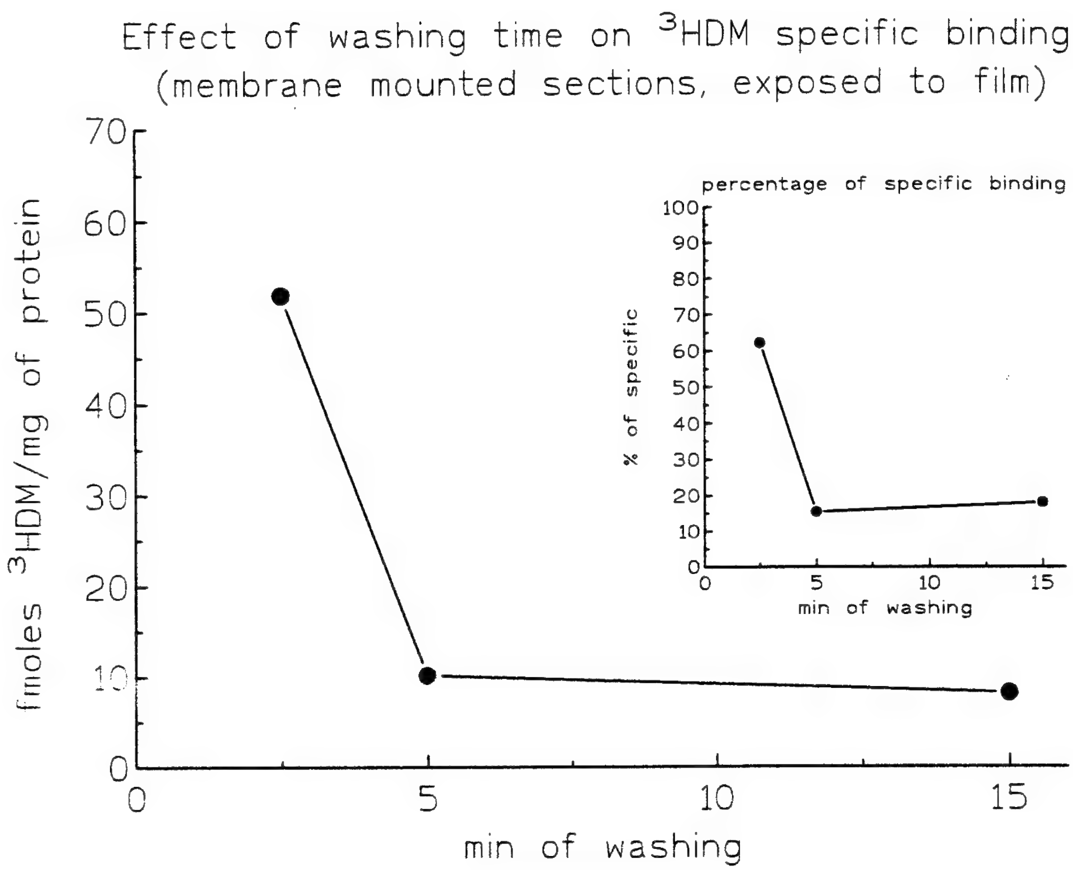
unfilled circles = total
filled circles = nonspecific
stars = specific

Fig. 18



Effect of washing time on ^{3}HDM binding
4°C, sodium phosphate 50 mM, pH 7.4
fmoles ^{3}HDM/section
squares= non specific binding
circles= Total binding
min of washing
specific binding
percentage of specific binding
% of specific
min of washing
fmoles ^{3}HDM/section
min of washing

Fig. 19



Effect of washing time on ^{3}HDM specific binding
(membrane mounted sections, exposed to film)
fmoles ^{3}HDM/mg of protein
70
60
50
40
30
20
10
0
0
5
10
15
min of washing
percentage of specific binding
% of specific
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
min of washing

Fig. 20

4.2.1.2 Optimization of binding parameters

Sections were incubated with 1 nM^{3}HDM in sodium phosphate buffer 50 mM pH 7.4 at 4° C for up to 6 h. Results are shown in Fig 18. Levels of specific binding reached the equilibrium plateaux after 48 min of incubation. The incubation time selected for further studies was 60 min.

The effect of different washing times was studied in fig. 19: specific binding remained fairly constant between 5 and 15 min of washing, while the non-specific binding was reduced 6-fold in the same interval.

Since previous studies with rat brain showed that the counts associated with the sections were not always the same when determined by liquid scintillation and autoradiography, we decided to test the effect of three different washing times (2.5, 5, 10 min) on large membrane-mounted sections of a human brain cortex` hemisphere. The effect of washing time can be seen in fig. 20: the percentage of specific binding for the washing times 5 and 15 min were much lower in these membrane-mounted sections than in these which were previously glass-mounted. The washing time of 2.5 min showed the optimal percentage of specific binding and signal definition. This time was selected for further studies.

5 Discussion

One of the most striking results of this study is that one component of DM binding to rat brain is sodium-dependent. This sodium-dependent binding of DM, which represents 90% of binding to areas such as MM, VTA and DR, was not inhibited by (+)PPP but was inhibited by DPH and PX. This would indicate that this binding site is not to a σ site.

The distribution of the sodium-dependent binding we detected in rat brain did not match with the binding profile of DM in guinea-pig brain (in sodium phospate buffer) reported by Canoll et al. (1989).
Binding of DM to rat brain in the presence of sodium in the present study was high in the midbrain, at the level of the MM, VTA, DR, CG, SC, with medium levels in the septal nuclei, thalamus and inferior colliculus. Very low binding was detected in the molecular layer of the cerebellum and in the DG. In comparison, studies in guinea-pig brain (Canoll et al., 1989) showed that DM binding to guinea-pig CER, CA3 and DG was as high as binding to MM, while binding to this nucleus was only half of that to DR and CG.

While DM binding to guinea-pig brain (Musacchio et al., 1983) presented marked similarities with that of σ sites labelled by (+)PPP (Largent et al., 1986), the distribution we found in rat brain correlated well with that of 5HT uptake sites labelled with PX. No correlation with the distribution of binding in guinea-pig brain (Canoll et al., 1989) could be demonstrated (fig. 21).
In fig. 15 is shown the correlation between DM and PX binding in rat brain. The only noticeable difference between DM and PX distribution was an apparent high binding to MM, VTA, and P, but the overall correlation was highly significant ($p < 0.001$, ANOVA). The distribution and the binding characteristics of the DM high affinity binding site appears to be species-dependent.

Binding in the absence of sodium showed a much less discrete localization, the ratio between the highest and the lowest binding detected being only 2 fold.
The binding profile in the absence of sodium did not correlate with the distribution obtained in its absence: this lack of correlation would suggest the presence of at least two components of DM binding. In fact, if the binding was only due to a sodium sensitive population of binding sites, and the residual binding in tris HCl was due to sodium impurities in the buffer, the overall profile should have had a parallel shift towards

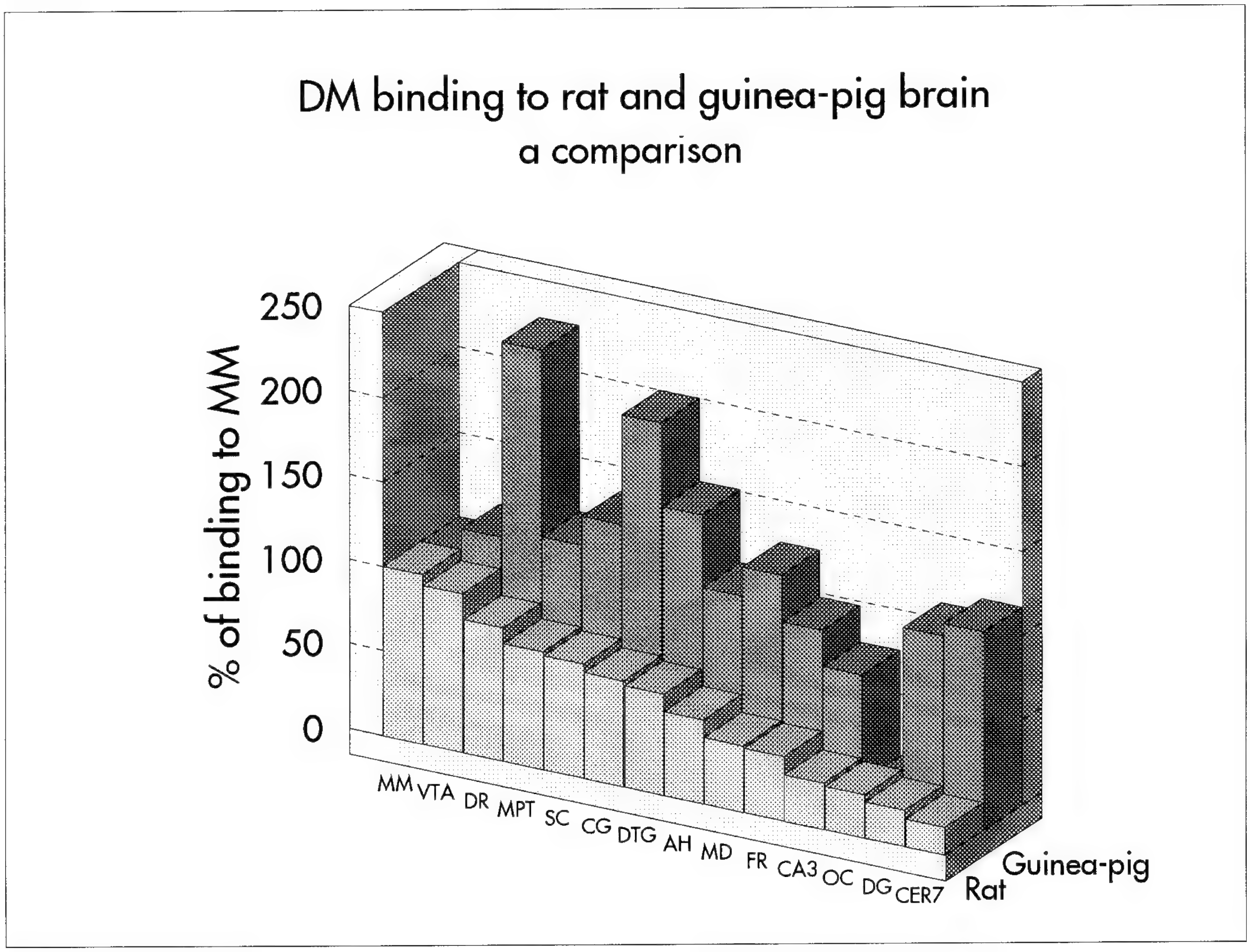

DM binding to rat and guinea-pig brain
a comparison
% of binding to MM
250
200
150
100
50
0
MM
VTA
DR
MPT
SC
CG
DTG
AH
MD
FR
CA3
OC
DG
CER7
Guinea-pig
Rat

Fig. 21

higher levels in the presence of sodium chloride.

Sodium-independent binding was not potentiated by DPH and was not inhibited by (+)PPP. It, therefore, did not share the same pharmacological properties of the DM_1/σ_1 sites that have been reported in the literature (Walker at al., 1990). The potentiation of sodium-independent binding by PPP at the highest concentration tested (176 nM) probably represents a non-specific effect of this substance, the action of which at DM_1/σ_1 sites has been reported to occur at much lower concentrations (Klein et al., 1992).

The different properties of dextromethorphan binding to guinea-pig and rat brain were not completely unexpected: Klein et al. (1992) have already demonstrated the different composition of dextromethorphan high affinity binding sites in the two rodents. In the guinea-pig the DM_1/σ_1 site is predominant whereas in the rat it is the DM_2 site that is prominent, representing more than 70% of the total binding (Klein et al., 1992; Zhou et al., 1991). The present study indicates that in rat brain, DM binding comprises a PPP-insensitive high affinity binding site that is sodium-sensitive, is inhibited by physiological concentrations of calcium and magnesium and is negatively modulated by phenytoin.

In one of the few autoradiographical studies on DM binding to guinea-pig brain, Canoll (1990) detected a differential effect of ropizine on binding in different areas of the encephalon.
Areas that in the present work showed the highest density of binding (DR, DTG, cortex) were the areas in which ropizine had an inhibitory effect (Canoll et al.,1990), conversely, areas in which the binding was greatly enhanced by ropizine showed very low levels of binding in our study.
These results taken together would therefore suggest that there are two high affinity binding sites for DM each with different pharmacological properties: the DM_1/σ_1 site, at which ^{3}HDM binding is displaced by (+)PPP and enhanced by DPH and ropizine (this constitutes the majority of binding in guinea-pig), and the DM_2 site, which is sodium dependent and negatively modulated by DPH.
Our study suggests that the DM_2 site is the most abundant in rat brain and that the distribution of this site has a resemblance to that of the 5HT uptake inhibitor, PX.
The identity between the binding of these two substances to rat brain was further supported by a mutual displacement study. Not 28only was the binding of DM inhibited by PX (10-100 nM), but also the binding of ^{3}H-PX (1 nM) was inhibited by DM 40 nM.
This mutual displacement rules out the interaction of these two

substances at a non-specific site and support the hypothesis that DM in the presence of sodium may bind to the 5HT transport system.

An experiment showing the lack of inhibition to DM binding by two cyt P450 inhibitors: SKF 525-A (proadifen) and GBR 12909 (preliminary data) suggests that the interaction of these two substances at the level of cytochrome P450, the enzyme responsible for the metabolism of both drugs, is quite unlikely. However, this preliminary data does not rule out the interaction of these two substances at a different binding site on the same enzyme.

At a more functional level, even though there is no overlapping between the clinical indications of PX and DM (Caley & Weber, 1993; Tortella et al., 1989), many preclinical studies (Sinclair, 1973) and some report on apparently idiosyncrasic reactions in humans (Nierenberg et al., 1993) suggest a probable common mechanism and possibly indicate new potential clinical indications for DM.

Currently, DM is widely used as an antitussive in many "over the counter" preparations, but it has also been reported to have anticonvulsant (Loscher & Honack, 1993; Apland & Braitman,1990) and anti-ischemic properties (Prince et al., 1988).

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PX has been recently approved for the treatment of depression and in anxiety, where it shows an efficacy comparable or slightly greater than other 5HT uptake inhibitors but presents fewer side effects and improved patient compliance (Caley & Weber, 1993). The role of serotonin in antitussive mechanisms in the rat has been investigated in several studies by Kamei and colleagues which have demonstrated:

i) potentiation of the antitussive activity of dehydrocodeine after 5HT depletion and subsequent sensitization of the animals to the effect of 5HT (Kamei et al. 1988)
ii) antagonism of the antitussive effect of non-opioid antitussives including DM by 5HT1A antagonists (Kamei et al. 1991)
iii) an increase in the release of 5HT in the NST by DM (Kamei et al. 1992).

All of these indications support a primary role for 5HT in the mechanisms affected by commonly used antitussives.
Buus-Lassen in 1977 reported a potentiation of the anticonvulsant effect of 5-hydroxytriptophan (5HTP) in the maximal electro-shock (MES) model in mice by PX, which also exhibited a significant anticonvulsant activity when administered alone (Buus-Lassen 1977).

It is therefore evident from these studies that PX can share some of the pharmacological properties of DM.

In comparison, even though there is no evidence of an antidepressant activity of DM, this drug has been reported to inhibit 5HT uptake in human platelets (Ahtee 1975) and prevent the short term toxicity of PCA in rats (Henderson 1992), both of which have indices of 5HT uptake inhibition in neurons.

All of these data suggest that DM and PX may have common mechanisms of action underlying part of their individual pharmacological properties.

Release studies to elucidate the effect of DM on 5HT release in the CNS, and some behavioural evidence for an antidepressant activity for DM are required to clarify the relationship between these two drugs.

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